AD_____

Award Number: DAMD17-98-1-8609

TITLE: The Role of Cumulative Genetic Defects in NF1 Tumorigenesis

PRINCIPAL INVESTIGATOR: Margaret Wallace, Ph.D.

CONTRACTING ORGANIZATION: The University of Florida
Gainesville, Florida 32611-5500

REPORT DATE: October 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020416 134

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE October 2001	3. REPORT TYPE AND DATES COVERED Final (1 Oct 98 – 30 Sep 01)	

4. TITLE AND SUBTITLE
The Role of Cumulative Genetic Defects in NF1 Tumorigenesis

5. FUNDING NUMBERS
DAMD17-98-1-8609

6. AUTHOR(S)
Margaret Wallace, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Florida
Gainesville, Florida 32611-5500

E-Mail: peggyw@cmg.health.ufl.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of this work is to study the genetic basis of tumor pathogenesis in neurofibromatosis type 1 (NF1). This work tests the two-hit hypothesis in NF1 tumors (benign neurofibromas and MPNSTs), the involvement of the TP53 gene in the tumors, and whether other loci contribute to the tumor formation. A large set of human primary tumor samples has been collected, with development of cell culture models from these tumors. One accomplishment has been discovery that the two-hit mechanism operates in at least a large proportion of neurofibromas, and that there is a genetically abnormal Schwann cell component in these tumors. Both germline and somatic NF1 mutations have been found, and we have evidence for somatic isodisomy as the mechanism for loss of heterozygosity. The TP53 gene has been found to be implicated only in the malignant tumors. The NF2 gene has allelic loss in some MPNSTs and plexiform tumors, with testing in dermal tumors and schwannomas underway. Substantial data from broader surveys of other genes (differential display, cDNA array, mutation screening) are under analysis. These data are important in the development of rational therapies to prevent or halt progression of neurofibroma and MPNST growth.

14. SUBJECT TERMS
Neurofibromatosis, NF1, tumor, genetics, Schwann cell, neurofibroma, plexiform, loss-of heterozygosity, antisense, mutation, gene expression

15. NUMBER OF PAGES
32

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a frequent autosomal dominant disease that displays variable expressivity, and is due to a mutation in the NF1 gene on chromosome 17. The features involve localized overgrowths or abnormal growths of neural crest-derived tissues. Thus, NF1 is characterized by abnormal cell proliferation, particularly evident in the formation of benign neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs). Neurofibromas contain mostly Schwann cells, which are thought to be a key cell type in these tumors. Nearly all NF1 patients develop dermal neurofibromas (small tumors in or under the skin on peripheral nerve twigs), about 40% develop plexiform tumors (larger, typically arising on deeper nerves), and about 5% develop MPNSTs (these usually occur within a previously-existing neurofibroma). Presently there are no known measures for the prevention of NF1 tumor development, and treatment of these tumors using standard therapies has not proven to be particularly effective because this is a lifelong progressive disorder and complete removal of the tumor sacrifices the attached nerve. Understanding of the progression of normal cells to a benign tumor and ultimately to malignancy is lacking in NF1; such knowledge would be invaluable for the development of preventive strategies, diagnostic tools and therapeutic approaches. Our work involves investigating the two-hit hypothesis in neurofibromas, which is the theory that the originating cell of a tumor contains the inherited/germline NF1 gene mutation plus a somatic mutation on the remaining allele, resulting in functional inactivation of NF1 and its protein. Due to the large size of the NF1 gene (60 exons, encoding 2818-amino acid protein), and the wide variety of NF1 mutations, detection of both germline and somatic NF1 mutations is challenging. A variety of genetic methods are being used to search for abnormalities in NF1, TP53, and other tumor-related genes, as well as screen for genetic and/or regulatory changes that might involve yet-unidentified genes, through several more global genetic analyses. Another aim is to study the functional effect(s) of NF1 and TP53 inactivation in Schwann cells through the use of antisense inhibition technology. The material for this work consist of solid tissue and cell cultures of NF1 tumors contributed by patients, which has provided and excellent resource for these and collaborative investigations. The combined use of these complementary approaches will lead to a more comprehensive understanding of the pathogenesis of tumors in NF1, to provide targets for future therapies.

<u>Technical Objective A</u>. Establish that NF1 Tumors Conform to the "Two-Hit" Hypothesis for the NF1 Gene.

<u>Progress on Task A1</u> (months 1-36): Continue/complete preparation of samples (DNA and RNA) from the current solid tissue and cultures. Continue subculture of tumoral Schwann cells.

Completed. We have prepared RNA and DNA from all of the solid tumors and cultures established too this point. In this current year we received tumor and blood samples from 5 NF1 patients (total of 12 tumors), along with blood samples from several unaffected relatives. Despite reducing our activity to recruit new samples, a number of patients continued to contact us to donate tissue (through word of mouth), which we accepted if we couldn't successfully refer to other labs interested in samples. This year we also obtained fresh tissue from five non-NF schwannomas (from one patient) for control/comparisons. Schwann cell enrichment through culturing was accomplished for 1 dermal neurofibroma and 1 plexiform neurofibroma (and 2 of the schwannomas, although after several passages we note that these cultures convert from Schwannian to more fibroblastic). We are also culturing Schwann cells from a normal (non-NF1) nerve for an additional control sample. We included the new samples in the analyses below when possible. We published our work establishing neurofibroma cultures and analyzing them at the protein level for neurofibromin (Muir et al., 2001, see appendix). We are also sharing these resources with other investigators (including some DOD-NF program funded investigators): Dr. Lynn Fieber (Univ. of Miami), Dr. Karen Stephens (Univ. of Washington), Dr. Yi Zhong (Cold Spring Harbor), Dr. Nancy Ratner (Univ. of Cincinnati).

<u>Progress on Task A2</u> (months 1-12): Complete intragenic loss of heterozygosity (LOH) studies on all available tumors; complete studies to outline extent of NF1 deletions.

Extended, nearly complete. LOH analyses have been extended throughout the project to include new tumor samples and culture DNAs. Our work through 1999 was published in 2000 (Rasmussen et al., 2000) in which 5% of dermal, 40% of plexiform neurofibromas (solid tissue only), and 60% of MPNST samples showed LOH in the NF1 gene region. We now have 14 NF1 intragenic markers working in the lab (7 restriction fragment length polymorphisms (RFLPs), 7 microsatellites), as well as some markers across the p and q arms to help delineate regions of LOH. During this past year we have done additional LOH work on new samples, mostly using informative RFLPs as an initial scan for LOH across the gene (ex. 5/RsaI, ex. 13/Tsp509I, EVI2B/EcoRI in intron 27b, and 3'UTR (base 10647/BslI)). This work is not quite complete, but combined with our previous work we now have found loss of one copy of the NF1 gene in the following solid tissue samples: 17/62 dermal neurofibromas, 14/45 plexiform tumors, and 5/7 MPNSTs (solid tissue, except 2 MPNSTs exist only as culture). An undergraduate student is finishing this work on the current specimens, as part of her research thesis during our no-cost extension year. Thus far, 2 dermal Schwann cell cultures show "purer" (but not complete) LOH than the primary tumor, supporting the notion that they are enriched for tumorigenic cells but also contain some normal Schwann cells. Among the plexiform tumors, relatively pure Schwann cultures exist for 21 samples, with one of these being from a patient with a germline NF1 deletion (and so not suitable for LOH analysis). Of the remaining 20, LOH is found in both the cultures and primary DNA for 4 samples,

with the cultures not being "pure," similar to what was observed in the dermal cultures. These are consistent with Western blot results (the purest sample showing no neurofibromin, the other three having some neurofibromin). MPNST cultures show complete LOH and are neurofibromin-deficient.

An offshoot of this project was a Southern blot analysis of LOH-positive samples, with consecutive probing of the blot with a di-zygous control probe and a polymorphic NF1 probe. The goal, along the lines of understanding 2nd-hit mechanisms in NF1, was to determine whether the allele loss was truly a deletion (hemizygosity) or a deletion with replacement of the sequences from the germline-mutant chromosome (isodisomy). We decided to look for this since Dr. Karen Stephens and Dr. Kevin Shannon had found this situation in NF1 leukemias (Stephens et al., 1998), with apparently common breakpoints near the ends of 17q. Our first analysis, this year, studied three tumors with strong LOH, two with weak LOH, and several with no obvious LOH (as well as a germline normal and germline large deletion cases, with known heterozygotes and homozygotes). The control probe was a non-polymorphic sequence from chromosome 15 as a source of normalization factor (DN34), and the polymorphic probe within NF1 was EVI2B (producing an EcoRI RFLP, thus EcoRI was used for the Southern blot digest). Data were gathered on a Molecular Dynamics Storm phosphorimager, and the intensities clearly support the notion that the remaining allele (containing the germline mutation) is present in 2 copies in the strong-LOH tumors (which were all plexiforms). The weak LOH tumors also yielded numbers for the germline copy that were greater than hemizygous, but not as strong (not clearly di-zygous, but in that direction) (probably related to amount of contaminating normal tissue in the tumor; these were dermal tumors). These data suggest that isodisomy is acting in benign neurofibromas, although we know from previous data mapping range of LOH that the isodisomic region must vary, in contrast to leukemias. We plan to do one more Southern blot with more samples and controls and then publish our data. Of note is that this phenomenon was very recently also identified in neurofibromas by Serra et al. (2001a). Of interest also is the fact that dermal neurofibromas rarely contain cytogenetic aberrations, based on our work (Wallace et al., 2000), and isodisomy would be an explanation for this. This finding is of importance in understanding neurofibroma pathogenesis since there may be a significant biochemical difference between a tumor hemizygous for 50 genes versus one that is di-zygous for the same allele of the same genes.

Progress on Task A3 (months 4-12): Complete NF1 protein truncation test (PTT) studies on all tumors for which NF1 LOH was not found to identify somatic mutations; complete NF1 PTT on all blood samples to identify germline mutations; characterize specific mutations (germline and somatic) on cases in which PTT has identified truncated proteins.

In progress. As described previously, many solid tumor RNAs were found to be of insufficient quality to yield unambiguous PTT results. Previously we had 4 PTT-positive tumor samples, with 3 reflecting the germline mutation (the fourth was ambiguous and probably really negative; we have been unable to find any mutations in the exons encoding that segment). A new round of PTT analysis is underway using tumor culture RNAs and blood RNAs, using modifications based on new commercial products and published work (e.g. Messiaen et al., 2000). For cultures grown this past year, we extracted

RNA from some plates cultured with puromycin, to enrich for transcripts containing truncating mutations; we and collaborators in Belgium (Messiaen et al., 1999, 2000) established that this treatment reduces nonsense-mediated decay in the NF1 transcript and therefore increases the likelihood of obtaining a positive PTT result (for germline or somatic mutation). Data from this work are imminent. We have also gathered substantial additional experience in interpreting direct PCR sequencing results on heterozygous templates, which should simplify determination of underlying mutation when sequencing the RT-PCR products after finding a positive PTT (originally we proposed cloning and sequencing, but this should not be necessary now).

One issue that, together with our experience suggests that the PTT is also not an optimal test, is the discovery of aberrant splicing in the NF1 gene due to sample handling. This was originally discovered by us (Wallace et al., 1998 abstract; Messiaen et al., 1999), but was first published in manuscript form by several other groups (Ars et al., 2000; Wimmer et al., 2000). We have been characterizing these forms, which are consistently missing partial or whole exons. These are related to exposure of blood to room temperature conditions for at least an hour; this is avoided with immediate processing or immediate refrigeration. In addition to those published, we have found several other forms and are within a couple of weeks of submitting the data as a manuscript. This would be the first report of a systematic search of the NF1 gene for aberrant splicing across the entire open reading frame, including in multiple tissues, and the main significance of this work is that these aberrant forms can lead to false positive PTT tests (in fact we give an example of this based on our own mutation work), and that there are not likely to exist any significant other alternatively spliced NF1 exons other than those already identified (exons 9br, 23a, 48a).

Because of the above pitfalls with the PTT, we have been moving efforts to employing single stranded conformational polymorphism (SSCP) analysis of the most frequently-mutated exons (e.g. 4b, 7, 10a, 10b, 16, 29, 31, 37) in tumors. Also, based on a paper last year (Eisenbarth et al., 2000, the first to publish more than one subtle somatic NF1 tumor mutation) which found nonsense mutations in most of the 7 neurofibromas studied, we screened for CpG and CpNpG transition nonsense mutations via looking for altered restriction enzyme analysis patterns. Restriction digests were employed to test for 23 different CpG nonsense mutations (covering 18 exons), similar to that done by us for germline mutations (Krkljus et al., 1997), and all 33 tumor DNAs proved negative for these mutations (suggesting no germline or somatic mutations). The tumor DNAs were mostly plexiform tumors (non-LOH), and we optimized for digestion and used polyacrylamide gels to increase our sensitivity of detecting even faint bands since the tumors are heterogeneous and somatic mutations might be present in a low frequency of cells used to make the DNA. The SSCP analysis, however, has been productive: 68 tumor samples are under analysis for 23 exons, and 15 mutations (mostly somatic, mostly 1-4 bp deletions, scattered across the gene) have been characterized, along with detection of 4 rare neutral variants. This work is substantially completed (half the samples still need 10 exons analyzed). The method uses a bipartite system where two SSCP gels are run—one regular native 10% acrylamide, and the other 10% acrylamide + 10% glycerol, both run at room temperature. Both types of gels complement each other in terms of detection abilities and so we continue to run both for each PCR. Since these are silver stained, we are finding that we have sufficient sensitivity to detect even faint abnormal bands and can identify the underlying mutations through direct sequencing or cloning

and sequencing. In two such faint cases we've detected pathogenic somatic mutations which represented only 1/12 clones. The somatic mutations have all been predicted to result in a truncated protein, except for one missense, but only includes one obvious splicing defect, in contrast to the recent publication suggesting that splice mutations are common somatic NF1 mutations (Serra et al., 2001b). However, that publication failed to find the DNA-based mutation in a few of its samples and thus it is possible that artifactual splicing was causing a false positive result, which is why our splicing study above will prove useful. The identification of both the germline and somatic mutations in 15 tumors (dermal and plexiform) further supports the tumor suppressor two-hit hypothesis. Once the data have been completed (given our current rate, another 2-3 months should finish it and produce at least 6 other somatic mutations), we will combine it with PTT and latest LOH data and publish the work (the drafting of the paper is underway).

Also note that collaborator Dr. Karen Stephens (Univ. of Washington) is also testing most of the tumor DNAs for the large repeat-mediated gene deletion in tumors (using their PCR-based assay, Lopez-Correa et al. (2001)) but to date none have proven positive (except those from patients with large germline deletion).

Also, it is known from other tumor syndromes (for both benign and malignant tumors) that the mechanism of the second "hit" can be methylation-based silencing instead of a primary mutation. Thus, one graduate student is using the sodium bisulfite sequencing method to assay for CpG methylation in a set of LOH-negative plexiform neurofibromas at the promoter region (400 bp spanning transcription start, where the expected "normal" pattern of NF1 gene methylation has already been established in non-Schwann cells and crosses from normally methylated to normally unmethylated). Since two papers from the last 2 years failed to find methylation differences neurofibromas (mostly dermal, not very many samples) (Horan et al., 2000; Luitjen et al., 2000), we are focusing on plexiform tumors. The method is working well and thus far 3-10 clones have been sequenced for 10 tumor DNAs (6 solid, 4 culture). Very promising data have been obtained: 5 tumors (2 solid, 3 culture) show at least one abnormal clone (with conversion from non-CpG cytosines looking very good), and for those samples we are analyzing more clones to test for consistency. Some of the sites of methylation lie on putative transcription factor binding sites, as well, suggesting a functional effect. Analysis of the methylation pattern in normal Schwann cells is also underway, which will provide a better comparison for this work. Analysis of relative allelic transcript quantity (using the exon 5 coding region RFLP) will also lend data to test whether methylation might be causing a transcriptional silencing. These data, plus the identification of LOH and specific somatic mutations above, support the tumor suppressor two-hit hypothesis in at least plexiform tumors through different mechanisms of somatic inactivation. More interesting, methylation-based silencing is potentially reversible, and so establishment of this phenomenon might open new possibilities for treatment of tumors with this characteristic.

Progress on Task A4 (months 6-12): NF1 tumor samples and derivative cultures will be analyzed using immunocytochemical and Western blot analyses to determine if they are indeed devoid of neurofibromin.

This Task is in progress, to finish analysis of new samples from past 15 months. Previous samples were summarized in this year's publication (Muir et al., 2001, appendix)

of finding of neurofibromin-deficiency in 18 Schwann cell cultures derived from neurofibromas (both dermal and plexiform, by Western blot). The other cultures (which did not show such strong enrichment for Schwann cells, or seem to have a cell type that is not typical Schwann) do show some neurofibromin upon Western blot, which is harder to interpret due to the culture characteristics. There are now 6 more Schwann cell cultures to be analyzed by Western blot, which we will finish in the next month or so. We have also immunostained over 40 dermal neurofibroma and 30 plexiform NF1 neurofibroma sections with the Santa Cruz N-terminal polyclonal NF1 antibody (corresponding to a peptide encoded within exon 10a), and have found that all have at least patches of neurofibromin-deficient Schwann cells (some completely devoid of neurofibromin staining within the tumor section), and some of these data are included in the above paper. This is consistent with the genetic data supporting the two hit hypothesis in all NF1 neurofibromas. Of note is that neurofibromin Western blots always shows additional bands below the neurofibromin area, as has been observed by other labs using this and other antibodies. Thus, as we proposed, we have created of a new antibody to see if we can find one that is clearer on Western blots and useful for other applications. A peptide corresponding to a hydrophilic stretch of 14 amino acids encoded within exon 2 (thus, very N-terminal, residues 27-) was used to for monoclonal antibody production. Two positive hybridomas, both strongly reactive to the original peptide (ELISA), detect neurofibromin; one works well on Western (but still shows lower bands, NFn27a) and the other is not very good for Western but is very strong on immunohistochemistry (NFn27b). Furthermore, with remaining peptide, Dr. Muir had a polyclonal rabbit antibody made, which is adequate for Western (no better than Santa Cruz) and also stains fixed sections very well. With the DOD's permission, these antibodies are now being produced and licensed so that other researchers can access them. Dr. Karen Stephens (Univ. Washington) is also testing an aliquot for some of her NF1 work. We will employ these to help finish this last part of the project.

Progress on Task A5 (months 6-12): Complete immunocytochemistry analysis for neurofibromin protein of tumors/cell cultures; complete Western blot studies for neurofibromin on tumors/cell cultures.
    See Task A4.

Progress on Task A6 (months 1-36): Complete antisense inactivation of NF1 to model NF1 inactivation in NF1-relevant cells; perform tumorigenicity related assays in antisense inhibited cells.
    In progress. As discussed last year, oligonucleotide antisense inhibition was abandoned in favor of stable transfection with plasmid constructs. In the previous year, an antisense construct of 450 bp of human NF1 cDNA (corresponding to exons 4a-6) in an inducible Invitrogen expression vector failed to express and perform as hoped in rat Schwann cells as a test of the system. Thus, two new inserts, both of which contain the natural NF1 translation start sequences (one extends into exon 3, the other into exon 7), were amplified this year from rat RNA, and cloned into the same inducible transfection system. The transfection and selection took several months, and the protein and RNAs have been harvested. An initial Western (using the Santa Cruz antibody) was uninterpretable because the positive control did not work, and thus we plan to repeat the work using our newly-derived antibodies above as well as other commercial antibodies.

The RNA has been reverse transcribed and the PCRs will be performed shortly, to test for expression of (1) a housekeeping gene (GAPDH and/or actin and/or HPRT) to test for quality of RNA and RT reaction, (2) the endogenous NF1 transcript (using primers downstream from the construct), and (3) the construct specifically (using one primer in the transcribed vector sequence and another within the insert). It is hoped that one or both of these antisense constructs will substantially reduce endogenous transcription, to yield cell lines lacking NF1 to test for tumorigenic properties (as compared to/complementary to mouse embryonic Schwann knockout cells). Such data would also be convincing preliminary work for another grant to introduce the same relative antisense construct in human Schwann cells, and provide a resource for other work such as in vivo experiments (the inducing agent is can be fed to the mice). Since antisense approaches have not generally been reported for genes as large as NF1, it is possible that there are underlying mechanisms that may effectively prohibit successful antisense inhibition for this gene and tolerate some degree of double stranded RNA (perhaps supported by the fact that some tissues express both NF1 and embedded genes such as EVI2A, EVI2B, and OMGP simultaneously; and RNA-structure analytical programs show strong secondary nature in the NF1 transcript, with lots of stem/loops, data unpublished). With respect to this project, we will finish the analysis of the current antisense clones within the next few months, as a final goal.

Technical Objective B. Evaluate the Involvement of the TP53 Gene in NF1 Tumors.

Progress on Task B1 (months 1-6): Complete TP53 LOH studies on all available tumors.
    Completed last year (Rasmussen et al., 2000).

Progress on Task B2 (months 12-24): Complete TP53 sequencing on all tumors.
    Completed last year (Rasmussen et al., 2000).

Progress on Task B3 (months 12-36): Complete inactivation of TP53 by antisense technology in Schwann cells; perform transformation related assays in TP53 antisense inhibited cells.
    As described last year, this Task is not being pursued further since TP53 is only involved in MPNSTs. Furthermore, the NF1/TP53 cis double knockout animals (laboratories of Tyler Jacks and Luis Parada) provide an MPNST system that supplants the need for an antisense approach.

Progress on Task B4 (months 18-24): Perform immunocytochemistry studies for p53 on all tumors.
    Completed last year.

Technical Objective C. Examine the Involvement of Genes other than NF1 and TP53 in NF1 Tumorigenesis.

Progress on Task C1 (months 1-12): Complete cytogenetic analyses on neurofibroma and neurofibrosarcoma cell cultures.
    Completed last year (Wallace et al., 2000).

<u>Progress on Task C2</u> (months 11-12): Perform comparative genomic hybridization (CGH) on 5 cutaneous, 5 plexiform tumors and 5 neurofibrosarcomas.

As mentioned in previous years' progress reports, our original collaborator withdrew and we were not going to pursue CGH further. However, last winter we established a collaboration with Dr. Deborah Marsh (Kolling Inst. of Medical Research, Sydney, Australia) who does CGH for PTEN-related tumors such as phaeochromocytomas. We sent 20 tumor DNA samples (13 plexiform; 5 dermal; 2 MPNST)(most of which are culture DNAs, not solid tissue DNAs, some LOH + and some LOH--) and her lab is now analyzing them; thus the effort is out of our hands for now but we will at least derive some data from this original approach (which we can correlate with our other data such as LOH and cytogenetics) and that should result in a publication some time next year.

In progress: alternative approach, nearly completed. As described last year, as an alternative a different approach was being undertaken—cDNA expression array analysis. Nine plexiform tumor culture RNAs were hybridized to the Clontech Atlas Cancer cDNA array membranes, compared to normal Schwann cell RNA, to identify aberrantly expressed transcripts (the array has over 1100 genes known to be involved in cancer-related systems such cell cycle, proliferation, apoptosis, DNA repair, etc.). Another set of hybridizations was performed with 3 other samples but the controls were not consistent and so those hybridizations were not considered reliable. In addition, the U.F. Shands Cancer Center awarded us 5 Affymetrix Human Cancer Chips (plus quality control chips), and we analyzed 3 of the same tumor RNAs plus a new one (and the same normal Schwann cells as used for Atlas analysis). The Affymetrix chip contained match and mismatch oligos for over 2000 cancer-related genes (many the same as on the Atlas membrane, but some different), and is a more sensitive system because of its fluorescence-based detection. Data generation has been completed and the analysis is underway, nearly finished. The most obvious observations are that the tumor samples are not dramatically different than normal Schwann cells, and that there is substantial heterogeneity between samples (not unexpected, given that these are from different human beings). With the help of another lab here experienced at analyzing these data, fairly stringent criteria were set to eliminate false positive results, and the gene expression data were plotted to identify those genes most commonly altered with respect to transcript quantity (expression fold change) . The genes that came to light were: TIMP3, MMP11, MMP1, TBSP2, NOTCH4, P75, and c-MYC, although these were not always altered in the same direction in every tumor. All of the above genes have a known role in tumorigenesis or can easily have that inferred based on its function (e.g. Notch references (Berset et al., 2001; Jang et al., 2000). The Roche LightCycler (a real-time PCR machine) has been made available to us, which is what is being used to confirm the transcript differences through RT-PCR. Thus far, the NOTCH4 data look very consistent, and several others are being analyzed now. Of note, the arrays suggested that GAPDH and beta-actin might be altered in the neurofibromas and thus would not be good normalization controls for expression changes. Thus, the HPRT transcript is being used for normalization, based on recent reports that this gene's expression is relatively immune to tumor-related effects (Roche Diagnostics). We will try to run a dendritic program to see if the array results can break the tumors into most related sets, although this may not work given the small number of samples and the two different approaches.

11

However, overall the data suggest that multiple pathways can be involved in tumorigenesis, and these data will provide preliminary data for future grants to further investigate the roles of these molecules in NF1 tumors.

Although not assayed through the array system, we were also interested in whether neurofibromas expressed telomerase, known to be associated with the immortalization phenotype in cancers. RT-PCR primers were designed and a preliminary experiment using silver staining suggested that only MPNSTs express telomerase, not plexiform tumors. We hope to follow up on this with better controls and possibly collaborate with a lab to run the telomerase assay on MPNST cells. Since this molecule is a target in other cancers, characterization of its presence/absence in NF1 tumors will help determine if those therapies are appropriate for NF1.

Progress on Task C3 (months 13-24): Perform LOH studies for other tumor suppressor loci on NF1 tumors.

In progress, nearing completion. A microsatellite marker in NF2 has shown LOH in most MPNSTs and 2/29 informative plexiforms. Analysis in dermal tumors (particularly those showing NF1 LOH and thus known to be relatively pure), and non-NF1 non-NF2 schwannomas is underway and should be completed within a month or two. If NF2 LOH is found specifically in plexiforms and malignancies, it may suggest that loss of the NF2 protein may contribute to uncontrolled tumor growth and Schwann cell malignancy. The p16 (CDKN2A) and TP73 gene analysis was done last year on the tumors most likely to show abnormalities, plexiforms and MPNSTs, with no evidence of LOH. An RB1 marker is now working and is being genotyped on a number of tumors; thus far there is no evidence for LOH in informative samples (5 dermals, 5 plexiforms). Originally, 8 genes were planned for LOH analysis, but this has been curtailed and will end with RB1 (which should be completed by 2002) since the yield of data is much greater from the cDNA array analysis.

In looking at other genes that might be activated in neurofibromas rather than inactivated, a mutation analysis was also done to search for a specific activating mutation in the ERBB2 oncogene that is reported in connection with development of Schwann-based tumors in young rodents treated in utero with mutagenizing agents (reviewed by Nakamura, 1995). ERBB2 signals through the ras pathway, and this receptor may also help bind to ligands and transmit signals relevant to Schwann cell proliferation (e.g. glial growth factor 2). Thus, we screened for this mutation (by DNA PCR followed by sequencing) in NF1 and non-NF1 neurofibromas (and schwannomas), but failed to find any mutations (there are no reports of activating human ERBB2 mutations, just amplification of the gene in cancers such as breast). However, the PCR assay spanned a known coding region missense polymorphism that is associated with breast cancer, and thus we genotyped this RFLP in a set of NF1 patients vs. normal controls and were surprised to find that there seems to be an under-representation of the rare homozygous genotype in NF1 patients compared to controls and the literature. This has maintained statistical significance even as the sample numbers have been increased to over 200 each. These data were just presented at the American Society of Human Genetics meeting (Fishbein et al., 2001), and a manuscript is being prepared. The interpretation is not entirely clear but the relationship is intriguing—it could suggest that there is an increased mortality in NF1 patients with homozygous for the rare allele, which makes this a

possible NF1 modifier gene via biochemical pathways. We will analyze the phenotype data available to see if there are any trends in features/severity.

Progress on Task C4 (months 25-26): Perform second CGH series on tumors of interest (probably all MPNSTs, unless alterations identified in plexiforms in first series of CGH).
    See Task C2.

Progress on Task C5 (months 18-24): Submit samples for differential display (DD) analysis to Core Lab.
    This work is completed with 22 bands sequenced. Unfortunately the UF Differential Display core closed and handed over the last of the RNA dot blot work to us this past year, and we found that their chosen control (beta actin) was not ideal based on our experience with cDNA array. None of the genes identified by differential display also came up as the best candidates by cDNA expression arrays, or were on the array membranes/chips. The two sequences that held up consistently through the dot blot were ESTs, one of which is increased in quantity, and the other of which is decreased in tumors relative to normal Schwann cells, although not dramatically. Neither EST has a genome match with a known gene, but represent an open reading frame of over 100 bp, and thus these are likely true, novel genes. In addition, genes identified (but whose real changes couldn't be confirmed by the dot blot) were ARPP-19 (cAMP phosphoprotein, 19kD), adenosine A2b receptor (ADORA2), MIHD (inhibitor of apoptosis protein 1), calumenin, alpha3 type IX collagen, and nickel-induced gene CAP43. A few remaining RT-PCR experiments will be attempted to pursue these genes further (to get corroborating data, especially for the ESTs) but given the overall poor reliability of Differential Display data that has been increasingly recognized over recent years, we will likely drop this line of investigation and concentrate on the cDNA array work as preliminary data for future directions.

Progress on Task C6 (months 25-36): Characterize and analyze specific genes suggested by CGH and DD experiments.
    Being completed - see Task C4 and C5.

13

KEY RESEARCH ACCOMPLISHMENTS

<u>For Technical Objective A (establish two-hit hypothesis in NF1 gene in NF1 tumors):</u>
- Task A1: Completed, with collection of several other tumors and cultures this year.
- Task A2: Extended, nearly complete--NF1 LOH studies on latest samples are being finished, along with finishing work identifying somatic isodisomy in tumors showing LOH.
- Task A3: In progress, using PTT to analyze culture RNAs treated with puromycin to improve mutation detection rate, finishing SSCP tumor mutation study to compile final numbers and types of somatic mutations, preparing to publish findings about environmentally-induced aberrant NF1 splicing, and finishing methylation study in plexiform tumors (to follow up on positive findings).
- Tasks A4 and A5: In progress, finishing Western blot analysis of newest culture samples using commercial and our newly-created neurofibromin antibodies.
- Task A6: Analysis of final antisense transfections is in progress at protein and RNA levels.

<u>For Technical Objective B (study involvement of TP53 gene in NF1 tumors):</u>
- Task B1: Completed.
- Task B2: Completed.
- Task B3: Dropped.
- Task B4: Completed.

<u>For Technical Objective C (examine involvement of genes other than NF1 and TP53):</u>
- Task C1: Completed.
- Task C2 and C4: CGH had been supplanted by cDNA array analysis, however a new collaborator is now doing some CGH experiments. cDNA array data analysis is nearly complete.
- Task C3: In progress, LOH being finished for NF2 and RB1 genes, and ERBB2 data being analyzed in preparation for manuscript submission.
- Task C5 : Completed.
- Task C6: Final analysis of differential display data is being finished.

REPORTABLE OUTCOMES

1. The tumor Schwann cell culture technique, the finding of neurofibromin-negativity in cultures by Western, and the ability of these cultures to survive and grow as sciatic nerve xenoplants in scid (immunodeficient) mice was published this year (Muir et al., 2001). This latter finding was the basis for a DOD application with Dr. Muir as PI, to develop this mouse model, which is ongoing.

2. The tumor Schwann cell cultures were the basis for a new DOD Army NF Program Investigator Initiated grant that was funded this year and is about to begin (NF000016). Graduate Student Lauren Fishbein (MD/PhD student) is finishing her work on several aspects of this current grant and will begin working on the new DOD project, having just received an NIH Training grant award from UF (Cancer Biology Training Grant). She also submitted an NRSA to pursue this work, which is pending, and would supplant the Training grant.

3. Manuscript reporting the aberrant NF1 splicing data (including sequence on new isoforms) should be submitted within the next few weeks.

4. Substantial progress has been made in finding subtle somatic NF1 mutations and these will be incorporated into a tumor genetics publication to be submitted by summer 2002.

5. The isodisomy finding will be pursued further to complete the data sufficient for a publication, to be submitted by summer 2002.

6. A license to sell the monoclonal and polyclonal amino-terminus neurofibromin antibodies has been approved.

7. Methylation analysis of NF1 tumors has yielded positive data which will be finished and written up for submission prior to the end of 2002.

8. cDNA array data, possibly with differential display data, should be analyzed sufficiently to be submitted as a manuscript prior to the end of this no-cost extension period. These data were presented as a poster at the American Society of Human Genetics meeting this October by graduate student Susanne Thomson (Thomson et al., 2001).

9. ERBB2 mutation/genotyping data were presented as a poster by graduate student Lauren Fishbein at the American Society of Human Genetics meeting this October (Fishbein et al., 2001) and will be submitted as a manuscript within the next few months.

10. Some of the isodisomy and somatic mutation data were reported by Dr. Wallace at an invited talk at the NNFF International Consortium Meeting on the Molecular Biology of NF1 and NF2, in June 2001, at Aspen, CO. Dr. Wallace has been invited to co-chair this same meeting next June.

CONCLUSIONS

The data substantially support the two-hit hypothesis in NF1 tumors, specifically in a genetically-abnormal clonal Schwann cell population in neurofibromas, with somatic mutations ranging in types. This suggests that loss of neurofibromin is associated with tumorigenesis, although whether this is sufficient for human tumorigenesis is still not completely answered (until antisense experiments work). It is unclear whether having even just minimal functional neurofibromin is enough to offset tumor formation, and we may be able to use this project's resources to determine this. Splicing errors, for example, are often leaky and thus one would expect that tumors containing one or two NF1 splicing errors would still produce a trace of normal neurofibromin. This does not seem sufficient to stop tumorigenesis, however. But our work indicates that caution must be exercised when analyzing NF1 splicing. If true, though, replacement of neurofibromin itself may not be a good therapeutic target because it may have to be present at greater than minor levels to be effective, and replacement of this gene and/or protein is not feasible due to the size and complexity and the number/location of target cells. It is hoped that our mutation data will ultimately shed light on possible functional domains of neurofibromin, so that there might be compensation for one or more functions without the need to replace the whole molecule. The cytogenetic complexity and the finding of methylation abnormalities in the plexiform tumors suggests that these tumors are more abnormal than dermal neurofibromas, and it is a combined effect of a number of gene aberrations that support the unusual growth. Since plexiform neurofibromas are more medically significant than dermal and affect more patients than MPNSTs, our focus has been on this tumor type in most of the experiments. The approaches in our work are aimed at finding biochemical abnormalities in the different tumor types, by defining the pathway heterogeneity present in the population. Both issues are crucial to develop new therapeutic targets, and so our data, particularly regarding genes with altered expression, will be very helpful for future directions in biochemistry and pharmaceutics.

REFERENCES

Ars E, Serra E, de la Luna S, Estivill X, Lazaro C. Cold shock induces the insertion of a cryptic exon in the neurofibromatosis type 1 (NF1) mRNA. Nucleic Acids Res 28: 1307-12, 2000.

Berset T, Hoier EF, Battu G, Canevascini S, Hajnal A. Notch inhibition of ras signaling through MAP kinase phosphatase LIP-1 during c. elegans vulval development. Science 291:1055-1058, 2001.

Eisenbarth I, Beyer K, Krone W, Assum G. Toward a survey of somatic mutation of the NF1 gene in benign neurofibromas of patients with neurofibromatosis type 1. Am J Hum Genet 66:393-401, 2000.

Fishbein L, Thomson SAM, Eady B, Wallace MR. Association between c-erb-B2 codon 655 polymorphism and NF1. Am J Hum Genet 69(suppl): 649, 2001.

Horan MP, Cooper DN, Upadhyaya M. Hypermethylation of the neurofibromatosis type 1 (NF1) gene promoter is not a common event in the inactivation of the NF1 gene in NF1-specific tumours. Hum Genet. 107(1):33-9, 2000.

Jang MS, Zlobin A, Kast WM, Miele L. Notch signaling as a target in multimodality cancer therapy. Curr Opin Mol Ther. 2:55-65, 2000.

Krkljus S, Abernathy CR, Johnson JS, Williams CA, Driscoll DJ, Zori R, Stalker HJ, Rasmussen SA, Collins FS, Kousseff BG, Baumbach L, Wallace MR. Analysis of CpG C to T mutations in neurofibromatosis type 1. Hum Mutation, Mutation in Brief #129 (1997) on-line.

Lopez-Correa C, Dorschner M, Brems H, Lazaro C, Clementi M, Upadhyaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns JP, Marynen P, Stephens K, Legius E. Recombination hotspot in NF1 microdeletion patients. Hum Mol Genet 10:1387-92, 2001.

Luijten M, Redeker S , van Noesel M, Troost D, Westerveld A, Hulsebos T. Microsatellite instability and promoter methylation as possible causes of NF1 gene inactivation in neurofibromas. Eur J Hum Genet 8:939–945, 2000.

Messiaen LM, Callens T, Roux KJ, Mortier GR, DePaepe A, Abramowicz M, Pericak-Vance MA, Vance JM, Wallace MR. Exon 10b of the NF1 gene represents a mutational hot spot and harbors a missense mutation Y489C associated with aberrant splicing in unrelated patients. Genetics in Medicine, 1:248-253, 1999.

Messiaen LM, Callens T, Mortier G, Beysen D, Vandenbroucke I, Van Roy N, Speleman F, De Paepe A. Exhaustive mutation analysis of the NF1 gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects. Hum Mutat 15:541-555, 2000.

Muir D, Neubauer D, Lim IT, Yachnis AT, Wallace MR. Tumorigenic properties of neurofibroma Schwann cells. Am J Pathol 58:501-513, 2001.

Nakamura T. Genetic markers and animal models of neurocristopathy. Histopathol 10:747-59, 1995.

Rasmussen SA, Colman SD, Ho VT, Abernathy CR, Arn P, Weiss L, Schwartz C, Saul RA, Wallace MR. Constitutional and mosaic large NF1 gene deletions in neurofibromatosis type 1. J Med Genet, 35:468-471, 1998.

Rasmussen SA, Overman J, Thomson SAM, Colman SD, Abernathy CR, Trimpert RE, Roux KJ, Bauer M, Rojiani AM, Maria BL, Muir D, Wallace MR. Chromosome 17 loss of heterozygosity studies in benign and malignant tumors in neurofibromatosis type 1. Genes Chrom Cancer 28:425-431, 2000.

Serra E, Rosenbaum T, Nadal M, Winner U, Ars E, Estivill X, Lazaro C. Mitotic recombination effects homozygosity for NF1 germline mutations in neurofibromas. Nat Genet 28:294-297, 2001a.

Serra E, Ars E, Ravella A, Sanchez A, Puig S, Rosenbaum T, Estivill X, Lazaro C. Somatic NF1 mutational spectrum in benign neurofibromas: mRNA splice defects are common among point mutations. Hum Genet 108:416-429, 2001b.

Stephens K, Weaver M, Leppig K, Manuyama M, Side L, Davis E, Espinoza R, Le Beau M, Shannon K. Double mitotic recombination at common breakpoint intervals leading to interstitial isodisomy and LOH. Am J Hum Genet 63:A39, 1998.

Thomson SAM, Fishbein L, Muir D, Wallace MR. cDNA array analysis of NF1 plexiform tumor Schwann cell cultures. Am J Hum Genet 69(suppl):257, 2001.

Wallace MR, Rasmussen SA, Lim IT, Gray BA, Zori RT, Muir D. Culture of cytogenetically abnormal Schwann cells from benign and malignant NF1 tumors. Genes Chrom Cancer 27:117-123, 2000.

Wallace MR, Trimpert RE, Thomson SAM, Fishbein L, Abernathy CR. Cryptic out-of-frame NF1 exon splicing in neurofibromas. Am J Hum Genet 63(suppl):A90, 1998.

Wimmer K, Eckart M, Rehder H, Fonatsch C. Illegitimate splicing of the NF1 gene in healthy individuals mimics mutation-induced splicing alterations in NF1 patients. Hum Genet 106: 311-3, 2000.

APPENDIX
    Muir et al. 2001 reprint
    Human subjects renewal letter.

Tumorigenic Properties of Neurofibromin-Deficient Neurofibroma Schwann Cells

David Muir,* Debbie Neubauer,* Ingrid T. Lim,*
Anthony T. Yachnis,† and Margaret R. Wallace‡

From the Divisions of Neurology* and Genetics,‡ the Department of Pediatrics, and the Department of Pathology and Laboratory Medicine,† University of Florida Brain Institute and College of Medicine, Gainesville, Florida

Dermal and plexiform neurofibromas are peripheral nerve sheath tumors that arise frequently in neurofibromatosis type 1. The goal of the present study was to examine the tumorigenic properties of neurofibromin-deficient human Schwann cells (SCs) that were found to represent a subset of SCs present in approximately half of the total neurofibromas examined. Highly enriched SC cultures were established from 10 dermal and eight plexiform neurofibromas by selective subculture using glial growth factor-2 and laminin. These cultures had low tumorigenic potential in classical *in vitro* assays yet several unique preneoplastic properties were frequently observed, including delayed senescence, a lack of density-limited growth, and a strong propensity to spontaneously form proliferative cell aggregates rich in extracellular matrix. Western blot analysis failed to detect full-length neurofibromin in any of the neurofibroma SC cultures, indicating that neurofibromin-deficient SCs had a substantial growth advantage. Immunohistochemical staining of the originating tumors showed the majority were comprised principally of neurofibromin-negative SCs, whereas the remainder contained both neurofibromin-negative and neurofibromin-positive SCs. Lastly, engraftment of neurofibromin-deficient SC cultures into the peripheral nerves of *scid* mice consistently produced persistent neurofibroma-like tumors with diffuse and often extensive intraneural growth. These findings indicate that neurofibromin-deficient SCs are involved in neurofibroma formation and, by selective subculture, provide a resource for the development of an *in vivo* model to further examine the role of these mutant SCs in neurofibroma histogenesis. (Am J Pathol 2001, 158:501–513)

Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition with a high frequency of peripheral nerve sheath tumors called neurofibromas. Dermal neurofibromas usually develop during adolescence and adulthood. These small tumors involve terminal nerves and may be numerous, yet have no apparent risk of malignant transformation. In contrast, plexiform neurofibromas are usually congenital, typically involve deep or "named" nerves, can become very large, and may cause serious functional impairment. Because plexiform tumors often occur on critical nerves and are not discrete masses, surgical removal is rarely complete and recurrence is associated with increased morbidity and fatality. A recent study suggests that plexiform neurofibromas develop in the majority of NF1 patients.[1] Additionally, plexiform tumors may progress to malignancy, which occurs in an estimated 6% of NF1 patients.[2]

Unlike schwannomas, which consist predominantly of Schwann cells (SCs), neurofibromas show marked cellular heterogeneity. Nevertheless, SCs are the major cell type amplified in neurofibromas and typically comprise 40 to 80% of the tumor cells. Additionally, there is a substantial population of interspersed fibroblastic or perineurial cells, along with various vascular and inflammatory elements embedded in an extensive extracellular matrix.[3] Because of this cellular heterogeneity, the histogenesis of neurofibromas has been controversial. Although there is increasing evidence for a SC origin, some studies suggest the contribution of SCs and fibroblastic cells.[4–6] On the other hand, an emerging view proposes that all of the major cellular elements of neurofibromas are of SC lineage and that the fibroblastic/perineurial-like elements may be immature or variant SCs.[7–10]

NF1 is caused by disruptive mutations in the *NF1* gene, which encodes the GAP-related protein neurofibromin. Thus, all cells in an NF1 individual are initially haploinsufficient for neurofibromin activity(s). However, consistent with the tumor suppressor gene two-hit model, it seems that loss of function of the remaining *NF1* allele is associated with neurofibroma formation, as first observed by our lab as loss of heterozygosity.[11] Although there are several approaches to animal models of NF1, presently none exist in which neurofibromas can be readily induced using defined human cell populations deficient in neurofibromin. Gene targeting has been used to construct mouse strains harboring mutations in the *Nf1* gene.[12,13] The mouse knockout model, similar to the

human NF1 condition, involves only a single constitutional mutation and homozygous mice ($Nf1^{-/-}$) die during gestation. Despite the high level of conservation between mouse and human neurofibromin, it is clear that *Nf1* knockout mice are not prone to the formation of neurofibromas. Thus, the inadequacy of the heterozygous mouse model may be attributed to a low mutation rate of the remaining wild-type *Nf1* allele within the relatively short murine life span. Original studies by Martuza and co-workers[14] demonstrated the growth of minced human neurofibromas in the subrenal capsule and sciatic nerve of immunodeficient mice that retained their morphological features and genomic identities. Thus, the use of defined neurofibroma cell populations in animal models will greatly enhance efforts to understand the histogenesis of neurofibromas.

Neurofibroma SCs have invasive and angiogenic properties, suggesting that these are genetically altered cells with tumorigenic properties.[15,16] Additionally, cytogenetic studies show that plexiform neurofibromas harbor genetically abnormal SCs and strongly implicate these cells as the central component in the development of these potentially progressive tumors.[17] Recently, somatic loss of heterozygosity was found in SCs, but not fibroblasts cultured from a neurofibroma, suggesting that genetic alterations of the *NF1* gene in SCs are involved in the development of neurofibromas.[18] In a more comprehensive study, Rutkowski and co-workers[19] further demonstrated that neurofibroma-derived SCs typically lacked *NF1* mRNA whereas fibroblasts isolated from neurofibromas expressed the *NF1* transcript. In the present study, SCs subcultured from numerous neurofibromas were examined for neurofibromin expression and tumorigenic properties *in vitro* and after intraneural engraftment. Our findings strongly implicate neurofibromin-deficient SCs in the histogenesis of at least a subset of neurofibromas.

Materials and Methods

SC Culture

Normal Adult Nerve SCs

All specimens included in this study were obtained in accordance with protocols approved by the University of Florida Institutional Review Board. Human SCs were isolated from normal adult sural nerves by modifications of methods described previously.[20,21] Briefly, segmented nerve fascicles were cultured for 10 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5% calf serum, 2 μm forskolin, 25 ng/ml human recombinant glial growth factor-2 (GGF-2), and antibiotics (expansion medium). The tissue was then dissociated for 18 hours in medium 15% calf serum, 1.25 U/ml Dispase (Collaborative Research Inc., Bedford, MA), 300 U/ml collagenase (type XI; Sigma Chemical Co., St. Louis, MO) and antibiotics. The digested tissue was dispersed by trituration, passed through a 30-μm mesh nylon screen, and centrifuged (200 × *g*, 10 minutes). The cell pellet was resuspended (50 segments/2

ml) in medium containing N2 supplements[22] and 2 ml of the cell suspension was spread across the surface of a 75-cm^2 flask precoated sequentially with polyornithine (0.1 mg/ml) and laminin (10 μg/ml) (prepared as described by Muir[23]). After a 6-hour incubation, the medium was supplemented by the gentle addition of expansion medium (10 ml). The cultures were grown to near confluency and the SCs were isolated by differential detachment using mild trypsinization and gentle shaking. The highly enriched cultures were expanded in expansion medium in dishes coated with laminin only. All cultures were withdrawn from treatment with forskolin and GGF-2 before storage or use.

Neurofibroma SCs

All patients met recognized diagnostic criteria for NF1[24] and tumor specimens were characterized as neurofibromas by histopathological study. Patient ages ranged from 4 to 69 years (the majority were young to middle-aged adults) and the reasons for surgery included cosmetic, functional deficit, and tissue donation on NF1-related fatality. These factors were unrelated to the tissue culture outcome and phenotype. Any capsular material was removed and viable tumor isolated from surgically resected neurofibromas. Tumor tissue (1 cm^2) was finely minced and incubated at 37°C overnight in 10 ml of L-15 medium containing 15% calf serum, 1.25 U/ml Dispase, 300 U/ml collagenase, and antibiotics. The tissue was dispersed by trituration and strained through a 30-μm mesh nylon screen. The filtrate was diluted with L-15 and centrifuged (400 × *g*, 5 minutes). The cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5% calf serum, and antibiotics (standard medium), and cells were seeded into tissue culture flasks ($\approx$10^6 cells/75 cm^2. After 4 days, cultures were detached with trypsin/ethylenediaminetetraacetic acid and passaged 1:4. Half of the passaged cells were grown in standard culture conditions; these were later harvested and stored in liquid nitrogen. The other half of the cells were seeded in flasks precoated with laminin (10 μg/ml) and grown in standard medium containing GGF-2 (25 ng/ml). GGF-2 treatment caused rapid proliferation of Schwann-like cells (SLCs). During subsequent passage, SLCs were enriched further by differential detachment using mild trypsinization and shaking. For the specified cultures, the combination of preferential laminin attachment, differential detachment, and selective mitogen treatment with GGF-2 yielded highly enriched (95 to 99.5%) SLC cultures within 3 to 4 passages. All tumorigenic and protein expression assays were performed using cultures at passage 3 to 4 that had been withdrawn from GGF-2 for at least 2 days. GGF-2 was generously provided by M. Marchionni (Cambridge Neuroscience, Cambridge, MA).

Anchorage and Serum Requirements

The growth of SLC-enriched cultures was assessed in serum-free and unattached conditions. Early (passage 2

Table 1. Characteristics of Neurofibroma-Derived Schwann Cell Cultures

SC Culture	Culture type*	S-100/p75†	NFn SC culture‡	NFn tumor§
Derived from dermal neurofibromas				
SC+(cNF89.1)	type-2	+/+	−	n.d.
SC+(cNF93.1a)	type-4	v/v	−	−/+
SC+(cNF93.1b)	type-4	v/v	−	−/+
SC+(cNF96.5f)	type-2	+/+	−	−/+
SC+(cNF96.5g)	type-2	+/+	−	−/−
SC+(cNF97.2a)	type-2	+/+	−	−/−
SC+(cNF97.2b)	type-2	+/+	−	−/−
SC+(cNF98.4a)	type-2	+/+	−	−/−
SC+(cNF98.4d)	type-2	+/+	−	−/−
SC+(cNF99.1)	type-2	+/+		
Derived from plexiform neurofibromas				
SC+(pNF92.1)	type-3¶	+/−	−	−/−
SC+(pNF94.5)	type-2	+/+	−	+/−
SC+(pNF95.1)	type-2	+/+	−	−/+
SC+(pNF95.5)	type-3	+/±	−	+/−
SC+(pNF95.6)	type-3¶	+/±	−	+/−
SC+(pNF95.11b)	type-3¶	+/+	−	−/−
SC+(pNF97.9)	type-4	v/v	−	−/+
SC+(pNF98.3)	type-3	+/+	−	−/+

*Growth classification specified in Results.
†Immunocytochemical expression of SC antigens, S-100 (cytoplasmic), and p75NGFR.
‡Expression of neurofibromin (+ or −) by NF1 SC culture determined by Western blot.
§Neurofibromin immunoreactivity for originating tumor as described in Results.
¶Proliferate without GGF2.
v, varied with density; ±, heterogeneous.

to 4) cultures were maintained in standard medium and detached from the culture dish with 0.5 mmol/L ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS). To examine anchorage dependency, cells were seeded in poly(HEMA)-coated culture wells at a density of 10^5 cells/well in Joklik medium (to minimize cell aggregation) supplemented with 10% serum. To examine serum dependency, cells were seeded in culture wells coated with laminin-1 (10 μg/ml) at a density of 10^5 cells/well and grown in serum-free N2 medium supplemented with 1% heat-inactivated bovine serum albumin. Cell viability was assessed at 0, 24, and 72 hours using a Trypan blue dye-exclusion assay. Counting chambers of a hemocytometer were filled with the cell suspension and viable cells (dye-excluding) as well as nonviable cells (dye-absorbing) were counted.

Growth in Soft Agarose

Anchorage-independent colony formation was determined by growing cells in soft agarose as described by Neugut and Weinstein.[25] A thin base-layer of 0.9% agarose was allowed to solidify for 1 hour in culture wells. A single-cell suspension in standard medium containing 0.4% agarose was layered over the solid agarose base and allowed to solidify. The cultures were grown for 2 to 8 weeks and viable colonies consisting of >25 cells were scored by phase-contrast microscopy. Percent colony formation was calculated as: (number of viable colonies/total viable cells seeded) × 100%.

Subcutaneous Engraftment

All animal procedures were performed in accordance with approved IACUC protocols. NF1 SC cultures were tested for their ability to form tumors after subcutaneous injection in immunodeficient nude mice. Cells grown on laminin in medium containing GGF-2 were harvested by trypsinization and resuspended in Hanks' balanced salt solution. Numerous subcutaneous injections were made using 2 × 10^6 cells/site. Mice were examined for development of palpable tumors for at least 3 months. Thereafter, animals were euthanized and the injection sites were surgically exposed and examined for signs of tumor formation. Because there were no signs of tumor growth no histology was performed.

Nerve Engraftment

Human neurofibroma-derived SC cultures (Table 1) from cryopreserved stocks were grown on laminin for 4 days in medium supplemented with GGF-2. Dissociated cells were collected, rinsed thoroughly, and resuspended as a dense slurry in Hanks' solution. Young adult scid mice were anesthetized and sciatic nerves of both legs were exposed at mid-thigh. A cell suspension (5 × 10^5 in 4 μl) was gradually injected intrafascicularly in both nerves through a fine needle (35 gauge) attached to a Hamilton syringe. The site was closed in layers with sutures and the revived mouse returned to specific pathogen-free housing. At 1 to 8 weeks after implantation, the animals were sacrificed under anesthesia and the nerves (n = 4

for each culture) were removed and fixed by immersion in 4% paraformaldehyde. Nerve segments were embedded in paraffin and sectioned for immunohistochemical staining.

Immunohistochemistry

Neurofibroma Cultures

Monolayer cultures were examined for immunoreactivity with antibodies to the SC antigens S-100 (DAKO, Carpinteria, CA) (1/300) and the low-affinity nerve growth factor receptor (p75) (4 μg/ml, hybridoma 200-3-G6-4; American Tissue Culture Collection, Rockville, MD). Cultures grown on laminin-coated chamber slides were fixed with 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 20 minutes, then washed with PBS containing 0.5% Triton X-100. Nonspecific antibody binding was blocked with PBS containing 0.1% Triton and 10% normal serum (blocking buffer) for 1 hour. Primary antibodies were diluted in blocking buffer and applied to wells for 2 to 4 hours at 37°C. Bound antibodies were labeled with peroxidase-conjugated secondary antibodies for 1 hour at 37°C and chromogenic development was accomplished with 3,3'-diaminobenzidine-(HCl)$_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS. Bromodeoxyuridine (BrdU) incorporation *in vitro* and immunolabeling of BrdU-DNA were performed as described previously.[26]

Nerve Grafts

Sciatic nerves engrafted with neurofibroma-derived SC cultures were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), sectioned in paraffin, and stained with hematoxylin and eosin for routine light microscopic examination. To identify transplanted human neurofibroma-derived SCs, nerve sections were immunostained with polyclonal anti-GSTπ (DAKO) (1/100) (a human-specific antiserum to the ubiquitous cellular protein, glutathione S-transferase) and a monoclonal antibody to p75 (4 μg/ml, hybridoma 200-3-G6-4) (a primate-specific antibody to the low-affinity nerve growth factor receptor). Deparaffinized sections were pretreated with methanol containing 1% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. Nonspecific antibody binding was blocked with 10% normal serum in PBS containing 0.3% Triton X-100 for 60 minutes at 37°C. Primary antibodies were diluted in blocking buffer and applied to sections overnight at 4°C. Bound antibodies were labeled with biotinylated secondary antibodies for 4 hours at 37°C followed by the avidin-biotin-peroxidase reagent (DAKO) for 2 hours. Chromogenic development was accomplished with 3,3'-diaminobenzidine-(HC1)$_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS.

Neurofibroma Tissue Specimens

Portions of the primary tumor used for cell culture were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), sectioned in paraffin, and stained with hematoxylin and eosin for routine light microscopic examination. Sections were immunostained for neurofibromin with the NF1GRP(N) antibody (1 μg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against a peptide corresponding to residues 509 to 528 of the predicted *NF1* gene product. The specificity of antibody to this neurofibromin peptide was reported previously.[27] Serial sections were immunolabeled with polyclonal anti-S-100 (1:300, DAKO). Immunoperoxidase labeling with the avidin-biotin-peroxidase reagent was performed as described above, except to enhance neurofibromin staining, antigen retrieval was achieved by pretreating sections in 0.1% trypsin for 20 minutes at 37°C. Immunostained sections were lightly counterstained with hematoxylin. Negative controls used no primary antibody. Additionally, for the NF1GRP(N) antibody, preadsorption with a 10-fold molar excess of peptide antigen (SC-67P, Santa Cruz) was used to achieve complete blocking of neurofibromin immunoreactivity.

Western Immunoblotting

SC cultures were scraped from dishes and cell pellets were homogenized in ice-cold extraction buffer consisting of 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and complete protease inhibitors (Boehringer-Mannheim, Indianapolis, IN). The soluble fraction was collected by centrifugation (10,000 × g, 20 minutes) and then was made 2 mol/L in urea. The extract was concentrated and fractionated by ultrafiltration using a 100-kd cut-off membrane. Total protein content of the high molecular mass retentate was determined using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). Samples were mixed with sodium dodecyl sulfate-containing electrophoresis sample buffer containing 2 mol/L urea and 5% 2-ME and then heated to 80°C for 2 hours. Samples (100 μg) were electrophoresed on 4 to 15% polyacrylamide gels and electroblotted to nitrocellulose sheets in transfer buffer containing 0.1% sodium dodecyl sulfate. Blots were rinsed in water and fixed in 25% isopropanol/10% acetic acid. Nitrocellulose sheets were washed with 0.05 mol/L Tris-HCl (pH 7.4) containing 1.5% NaCl and 0.1% Triton X-100 and then blocked in the same buffer with the addition of 5% dry milk (blocking buffer). The blots were incubated for 2 hours with anti-NF1GRP(N) antibody (1 μg/ml) in blocking buffer. Bound antibody was detected by peroxidase conjugated swine anti-rabbit IgG (affinity purified, DAKO) diluted 1/2,000 in blocking buffer. Immunoreactive bands were developed by chemiluminescent methods (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Relative molecular mass was determined using prestained markers including myosin (205 kd). Control samples were similarly processed from cell pellets obtained from normal human nerve SC cultures and SC cultures derived from embryonic homozygous *Nf1* knockout mice.[12]
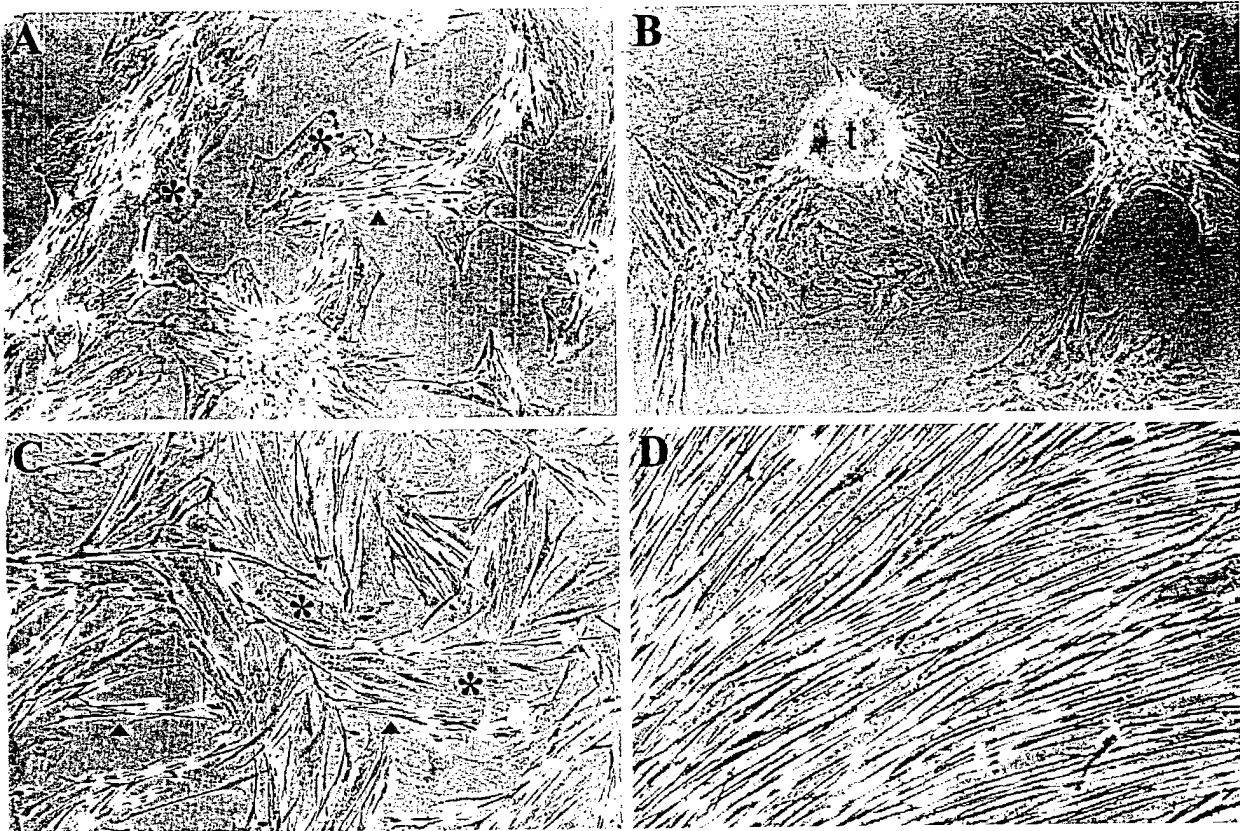
Figure 1. Subculture of neurofibroma-derived SCs. **A:** A typical primary neurofibroma culture containing mainly SLCs and FLCs. Seeded on tissue culture plastic, phase-bright SLCs (**arrowhead**) grew exclusively on patches of underlying FLCs (**asterisk**). **B:** SLCs proliferated in response to GGF-2 and formed dense cell aggregates (culture tumors, t) demonstrating their lack of contact-inhibited grow and continued association with the FLC sublayer. **C:** SLCs preferentially attached to a laminin substratum, which circumvented the association of SLC to the FLC layer and allowed expansion of the SLCs. **D:** A highly enriched SLC population was subcultured by growth on laminin in the presence of GGF-2 within three to four passages. Original magnification, ×200.

Results

### Culture of Normal Human Nerve SCs

SCs do not proliferate in response to standard serum-supplemented medium and, until recently, methods for isolating normal human SCs were mostly unreliable. Based on recent advances,[20,21] we investigated various means to enrich and expand SCs from adult human nerve segments. Successful enrichment of SC cultures and depletion of fibroblastic cells was readily achieved by treatment with the SC mitogens GGF-2 and forskolin combined with differential cell detachment and preferential growth on a laminin-coated substratum. After 3 to 4 passages under these conditions, hundreds of millions of SCs were obtained nearly free of fibroblast contamination from several centimeters of adult tibial nerve. These cultures were homogeneous and contained highly elongated SCs that stained intensely for S-100 and p75, recognized markers for cells committed to a SC lineage.[28] The specificity of S-100 as a marker for cultured human SCs is shown in Figure 3A. After enrichment and expansion, SC division rapidly decreased in the absence of GGF-2 and forskolin. Growth on a laminin substratum was required at all stages to improve attachment and to minimize cell attrition. SC expansion was limited to ~10 population doublings before senescence regardless of mitogen stimulation, confirming the earlier findings by Rutkowski and colleagues.[29]

Culture of Neurofibroma SCs

Our goal was the enrichment and characterization of SLCs from dermal and plexiform neurofibromas. Mono-layer cultures of neurofibromas were initiated by enzyme dissociation under standard culture conditions. The most frequent primary culture obtained from dermal and plexiform neurofibromas contained a sublayer of fibroblast-like cells (FLCs) admixed with numerous (20 to 60%) spindle-shaped SLCs (Figure 1). Numerous procedures to enrich and expand SLCs from the primary cultures were tested. It is notable that enrichment of SLCs was not improved by combined treatment with GGF-2 and agents that elevate cAMP. In particular, forskolin caused considerable heterogeneity in the SC population and hampered the development of the SLC cultures described below. Thus, unlike normal SC cultures, neurofibroma cultures were treated only with GGF-2 and were not exposed to forskolin.

Categorical responses to SC enrichment procedures (see Materials and Methods) emerged for the neurofibroma cultures. We defined four culture types based on prevalent cell morphologies, growth under standard cul-
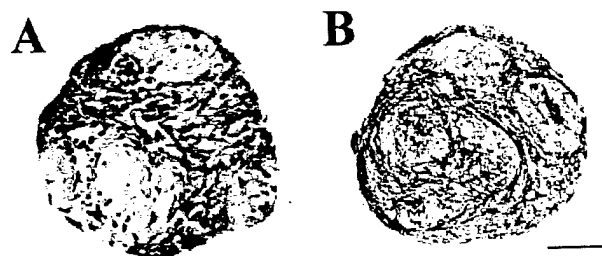
Figure 2. Neurofibroma SCs aggregated and formed culture tumors. Aggregates of SLCs, such as those shown in Figure 1B, were collected from early neurofibroma cultures and sectioned. **A:** H&E staining of culture tumors revealed areas of high cell density and cell-sparse regions of dense extracellular matrix similar to those seen in neurofibromas. **B:** Immunolabeling with laminin demonstrated a rich basement membrane network throughout the SC aggregates. Scale bar, 50 μm.

ture conditions, growth response to GGF-2 and laminin, and the onset of senescence. Findings were based on 40 robust cultures, 24 from dermal and 16 from plexiform neurofibromas.

A first type of neurofibroma culture (type-1) was distinguished by a poor response to the SC enrichment treatment with GGF-2 and laminin. These cultures (from dermal and plexiform tumors) were rapidly dominated by FLCs that often displayed abnormal growth characteristics (eg, rapid and protracted proliferation and loss of density-limited growth). The SLCs (immunopositive for S-100 and p75) failed to proliferate sufficiently and became increasingly diluted with repeated passage. Type-1 cultures accounted for 14 of 24 dermal and eight of 16 plexiform cultures, were deemed intractable to SLC enrichment, and were excluded from the analyses to follow.

A second subset, type-2 neurofibroma cultures, were amenable to enrichment of the SLCs. In most of these cultures the SLCs proliferated rapidly in the presence of GGF-2 and soon formed confluent islands on top of underlying FLCs (Figure 1A). Cultured on native tissue culture plastic, the SLCs attached exclusively to the FLCs. Despite the limited surface area of the underlying the FLCs, proliferation by the SLCs continued in the presence of GGF-2. As a result, the SLCs formed dense cellular masses or culture tumors (Figures 1B and 2A), indicating the absence of contact inhibition (density-limited growth) by neurofibroma SLCs. Immunolabeled sections of these culture tumors showed an extensive laminin-rich extracellular matrix (Figure 2B). These observations potentially relate to the adhesive mechanisms involved in the growth and development of neurofibromas.[30]

Growth on a laminin substratum, which circumvented the adhesion of the SLCs to the FLCs, was necessary for the subsequent enrichment and expansion of the SLCs (Figure 1C). Once outnumbered by the mitogen-driven SLCs, the FLCs were effectively diminished by differential detachment. Thereafter, the SLC population was readily enriched and expanded in large numbers within 3 to 4 passages (Figure 1D). Type-2 SLC cultures stained for S-100 and p75 and generally resembled highly spindled SCs obtained from normal nerve (Figure 3, A and B). However, purified SLCs from some neurofibromas, particularly those from plexiform tumors, were stubby and

less elongated (Figure 3C). The SLCs derived from dermal neurofibromas had a limited proliferative capacity similar to that of normal adult SCs (10 doublings) and became senescent thereafter. By comparison, the plexiform SLCs were less restricted and often were passaged >20 times before showing signs of senescence. All type-2 SLC-enriched neurofibroma cultures grew very slowly when withdrawn from GGF-2, but were stable for months on a laminin substratum. SLC-enriched type-2 cultures accounted for eight of 24 dermal and two of 16 plexiform cultures.

A third type of neurofibroma culture was obtained exclusively from plexiform tumors and contained SLCs with several preneoplastic properties. Type-3 SLCs expanded rapidly without close association with the FLC sublayer and were easily enriched to near homogeneity. Five type-3 cultures were established from plexiform tumors; SLCs in two cultures had a stubby, spindle shape whereas the other three cultures were multipolar or polygonal (Figure 3D). In each culture, nearly all cells were stained for S-100, whereas p75 expression varied. Three multipolar/polygonal type-3 cultures grew particularly well (doubling times 2 to 4 days) in response to serum and GGF did not further increase their growth rates. These cultures also showed protracted expansion (>20 to 50 passages). The occurrence of these preneoplastic properties is almost certainly indicative of genetic abnormalities originating *in vivo* because growth factor-independent proliferation and expansion beyond 10 passages were never observed in cultures of normal human SCs (also see Rutkowski et al[29]). Furthermore, the three GGF-independent type-3 cultures were derived from sizable recurrent plexiform neurofibromas.

A fourth subset of neurofibroma culture, at low density, were pleomorphic, lacy and phase-pale, and stained faintly, or not at all, for S-100 and p75. However, with increasing density they became elongated spindle cells and grew in parallel arrays. Remarkably, when grown to confluency, these cultures formed dense ridges of S-100-expressing SLCs (not shown). The appearance of these S-100-positive SLCs did not decrease after numerous passages but, instead, continued to increase in number with increasing culture density. These cultures were expanded extensively (>50 passages) before showing signs of senescence. These observations suggest that these cultures contained a poorly differentiated cell type capable of giving rise to a reversibly differentiated SLC component. This type of pleomorphic property also was observed in several neurofibrosarcoma cultures (not shown).

In summary, 40 primary cultures were established from 24 dermal and 16 plexiform neurofibromas and subcultured for enrichment of SLCs. Twenty-two of 40 cultures did not respond to SLC enrichment or were predominantly fibroblastic (type-1). Ten dermal and eight plexiform tumor cultures yielded enriched SLC cultures (type-2, -3, and -4) suitable for additional genetic and biological studies (listed in Table 1). The type-2 and type-3 cultures (eight from dermal and seven from plexiform tumors) were the focus of subsequent studies.
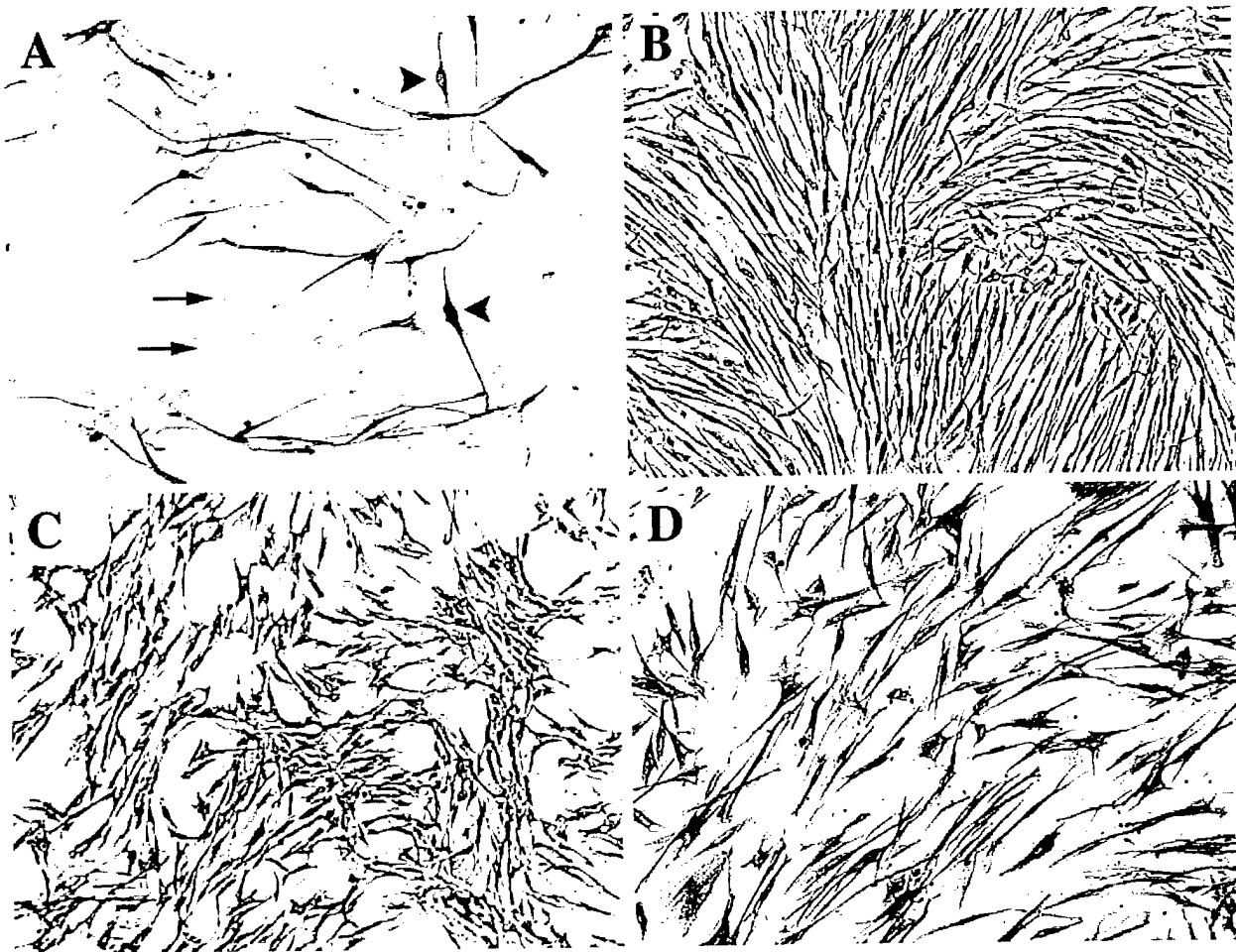
Figure 3. Morphology and S-100 immunostaining of nerve and neurofibroma-derived SC cultures. **A:** A primary culture established from normal adult human nerve, like those from neurofibromas, contained a mixture of S-100-positive SCs (**arrowheads**) and fibroblastic cells (**arrows**). SCs showed intense cytoplasmic S-100 immunoreactivity, while faint (if any) staining was visible in the nuclei of fibroblastic cells (contrast-enhanced image). After enrichment and expansion of SLCs from neurofibromas, the resulting SC cultures immunostained positive for S-100 yet often differed in morphology and growth properties (**B–D**). **B:** Type-2 cultures from dermal neurofibromas most often showed highly elongated spindle-shape cells similar to normal SCs. **C:** Type-2 and some type-3 cultures from plexiform neurofibromas generally contained multipolar and stubby spindled cells. **D:** Type-3 cultures were obtained exclusively from plexiform tumors and contained elongated but primarily polygonal cells. Original magnification, ×200.

Neurofibromin Expression by SCs in Neurofibromas

Before neurofibroma specimens were prepared for cell culture, representative specimens of tumor were fixed and processed for routine histology and immunohisto-chemistry. Paraffin sections were immunostained with an anti-neurofibromin peptide antiserum. Neurofibromin immunoreactivity in normal control tissues was similar to that reported by other laboratories.[31] In neurofibromas the SC elements were identified by their nuclear charac-teristics (elongated, wavy nuclei with pointed ends) and positive immunoreactivity for S-100 protein (Figure 4). Four basic patterns of neurofibromin immunoexpression in S-100-positive regions were observed: 1) neurofibro-min-negative ($-/^-$); 2) predominantly neurofibromin-neg-ative with focal areas of distinct positive staining ($-/^+$); 3) predominantly immunoreactive tumor with focal areas of distinct negativity ($+/^-$); and 4) neurofibromin-positive ($+/^+$). Immunoreactivity patterns for the surgical resec-

tion specimens corresponding to the SC cultures from which they were derived are summarized in Table 1. The majority (14 of 17) of the neurofibromas that gave rise to SC cultures were predominately neurofibromin-negative (designated $-/^-$ and $-/^+$ in Table 1). Moreover, more than one-half of these tumors were completely negative ($-/^-$), showing no neurofibromin immunostaining in any SC elements (Figure 4A). Furthermore, even in the few predominately neurofibromin-positive tumors ($+/^-$), many individual SCs were negative for neurofibromin (Figure 4C). In areas of positive immunoreactivity, tumor cells showed discrete, granular staining in the perinu-clear cytoplasm and in delicate elongated processes. In tumors with positively and negatively stained regions, the regions of neurofibromin nonreactivity were histologically similar to immunoreactive areas. Overall, neurofibromin immunoexpression in most neurofibromas that gave rise to a SC culture was very low (see Discussion). The finding of both positive and negative areas of neurofibromin im-munoreactivity in some neurofibromas could not be at-
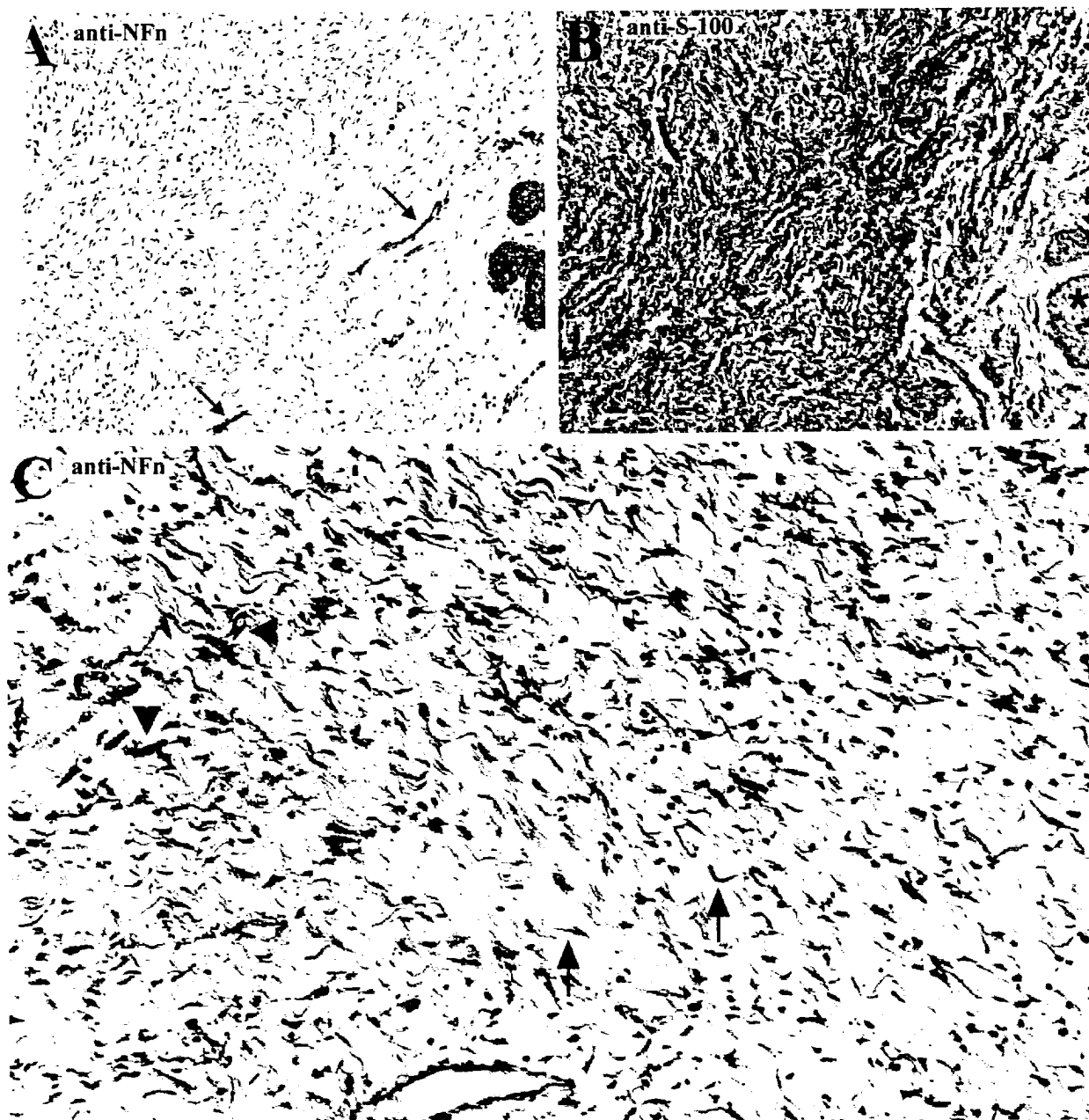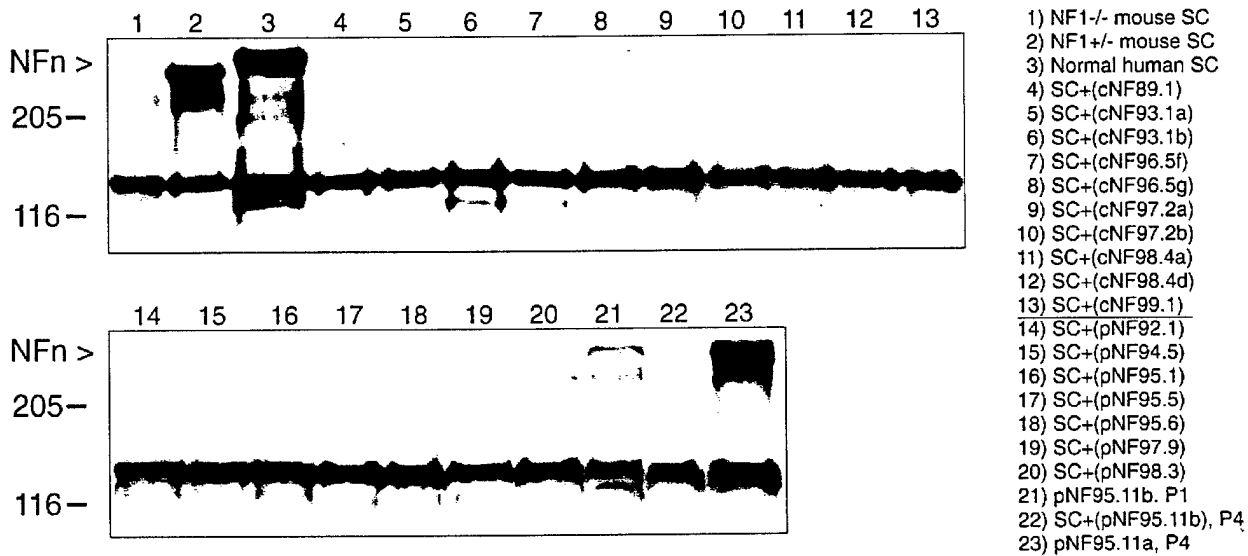
Figure 4. Neurofibromin immunoreactivity of SCs in neurofibromas. Tumor sections were immunoperoxidase stained for neurofibromin (A and C) and S-100 (B). A: Most neurofibromas that gave rise to SC cultures were either mainly or completely devoid of neurofibromin-positive SCs. Vascular elements (**arrows**) and sweat glands (**asterisk**) stained for neurofibromin and served as internal positive controls. B: A serial section of the neurofibroma shown in A showing strong and diffuse immunoreactive for the SC marker protein, S-100. C: A neurofibroma containing intermixed populations of neurofibromin-positive (**arrowheads**) and neurofibromin-negative SCs (**arrows**). In areas of positive immunoreactivity, tumor cells showed discrete, granular staining in the perinuclear cytoplasm and in delicate elongated processes. SC elements were identified by their nuclear characteristics (elongated, wavy nuclei with pointed ends) and positive immunoreactivity for S-100 protein. Sections were counterstained with hematoxylin. Original magnifications: ×200 (A and B), ×400 (C).

tributed to artifacts of fixation, tissue preparation, or regional differences in antibody concentration. Dermal and vascular elements were uniformly positive for neurofibromin in both immunoreactive and nonreactive regions of a given tumor (Figure 4). Also, tumors that were negative or that contained areas of neurofibromin nonreactivity were all strongly and widely immunoreactive for S-100 protein (Figure 4B).

Neurofibromin Expression by NF1 SC Cultures

We hypothesized that the abnormal growth properties of NF1 SC cultures, particularly the type-3 cultures, are the result of a severe deficiency in neurofibromin expression. Normal SCs and type-2, -3, and -4 NF1 SC cultures were examined for the expression of neurofibromin by Western immunoblotting. Results are shown in Figure 5. Antibody

1) NF1-/- mouse SC
2) NF1+/- mouse SC
3) Normal human SC
4) SC+(cNF89.1)
5) SC+(cNF93.1a)
6) SC+(cNF93.1b)
7) SC+(cNF96.5f)
8) SC+(cNF96.5g)
9) SC+(cNF97.2a)
10) SC+(cNF97.2b)
11) SC+(cNF98.4a)
12) SC+(cNF98.4d)
13) SC+(cNF99.1)
14) SC+(pNF92.1)
15) SC+(pNF94.5)
16) SC+(pNF95.1)
17) SC+(pNF95.5)
18) SC+(pNF95.6)
19) SC+(pNF97.9)
20) SC+(pNF98.3)
21) pNF95.11b. P1
22) SC+(pNF95.11b), P4
23) pNF95.11a, P4

Figure 5. Western immunoblot analysis of the SC cultures for neurofibromin expression. Culture cell pellets were extracted and a high molecular mass fraction was obtained by ultrafiltration (>100 kd). Samples (100 μg of total protein) were electrophoresed and transferred to nitrocellulose sheets. The blots were stained for neurofibromin using antibody NF1GRP(N) and chemiluminescent detection. Antibody specificity was demonstrated on extracts of SC-enriched cultures from embryonic *Nf1* knockout mice. Full-length neurofibromin was absent from homozygous cultures (*Nf1*$^{-/-}$) (**lane 1**), whereas heterozygous cultures (*Nf1*$^{+/-}$) (**lane 2**) expressed a predominant immunoreactive band-pair with a M_r = 225 to 250 kd, slightly smaller than the M_r = 240 to 260 kd neurofibromin bands produced by normal human SCs (**lane 3**). A band at 140 kd appeared in each sample that, although undefined, indicated the consistency of total protein loaded in each lane. Full-length neurofibromin was absent in extracts of the 10 dermal SC cultures (**lanes 4 to 13**) and eight plexiform SC cultures (**lanes 14 to 20 and 22**). Also shown is the original mixed primary culture pNF95.11b (**lane 21**) from which the type-3 SC culture, SC+(pNF95.11b), was derived (**lane 22**) and the type-1 culture, pNF95.11a (an earlier specimen from the same patient).

specificity was examined on extracts of SC-enriched cultures from embryonic *Nf1* knockout mice. Full-length neurofibromin was absent from homozygous cultures (*Nf1*$^{-/-}$) (lane 1), whereas heterozygous cultures (*Nf1*$^{+/-}$) (lane 2) expressed a predominant immunoreactive band-pair with a M_r = 225 to 250 kd (slightly smaller than that produced by human cells). A band at 140 kd appeared in each sample that, although undefined, indicated the consistency of total protein loaded in each lane. Extracts of normal human SC cultures (lane 3) contained a predominant neurofibromin-immunoreactive band-pair with a M_r = 240 to 260 kd. In contrast, full-length neurofibromin was absent in extracts of the 10 dermal SC cultures (lanes 4 to 13) and eight plexiform SC cultures (lanes 14 to 20 and 22). As an example, we also show the mixed primary culture pNF95.11b (lane 21) from which the SC culture, SC+(pNF95.11b) (lane 22), was derived. The first passage of the culture pNF95.11b contained an admixture of cells (including numerous FLCs and perhaps diverse SC lineages). A modest band-pair corresponding to full-length neurofibromin was observed in this culture extract (lane 21), indicating the genetic heterogeneity in this early culture. This finding is consistent with the pattern of neurofibromin immunostaining (+/–) observed in the originating tumor sections (Table 1). However, neurofibromin expression was undetectable in the derived type-3 SC culture (lane 22). The pNF95.11b cultures were established from a resection of a recurrent plexiform neurofibroma. Two years earlier, we established a culture from a specimen obtained from the initial resection. This culture, pNF95.11a, was type-1 (dominated by FLCs and intractable to SC enrichment) and expressed abundant neurofibromin (lane 23). Neu-

rofibromin content in this culture extract was presumably contributed by the large population of fibroblasts, but we cannot exclude the contribution by *NF1* heterozygous SCs. From these results we conclude that neurofibromin-deficient SCs have the best long-term growth advantages and that our subculture methods to enrich for SCs from neurofibromas are highly selective for neurofibromin-deficient SCs.

Properties of NF1 SC Cultures in Classical Tumorigenic Assays

Neoplastic and tumorigenic properties of type-2 and -3 neurofibroma SC cultures were examined in several classical tests, including assays of serum and anchorage dependence, colony formation in soft agarose, and subcutaneous tumor formation in immunodeficient mice. The proliferative properties (normal and abnormal) of these cultures were described in a previous section. Although not all of the NF1 SC cultures listed in Table 1 were tested repeatedly, findings of tumorigenic properties were consistent for all type-2 and -3 cultures from dermal and plexiform tumors. Notably, all type-2 and type-3 NF1 SC cultures showed properties similar to normal human SCs. First, the survival of the NF1 SC cultures and normal SCs was not highly growth factor-dependent, as 85 to 95% of cells remained viable in serum-free medium for at least 72 hours (compared to 95% survival of the highly tumorigenic RN22 schwannoma and C6 glioma cell lines). Proliferation was not observed in any of the SC cultures in the absence of serum. Second, the survival of the SC cultures was anchorage-dependent. Under nonadherent

conditions the percentage of viable normal SCs dropped below 40% after 72 hours. Similarly the survival of NF1 SC cultures was 20 to 40% after 72 hours in suspension culture (compared to >95% in the RN22 and C6 lines).

Colony formation in soft agarose is an indicator of high tumorigenicity and anchorage-independent growth, properties of transformed cells and some, but not all solid tumor cultures. Suspended as single cells in soft agarose, 72% of RN22 and 88% of C6 cells proliferated and rapidly formed colonies that become visible to the eye within 2 weeks. In contrast, multicellular foci of normal SC or NF1 SC cultures were rarely observed and none reached the 25-cell colony criterion, even after cultured for 2 months in agarose.

NF1 SC cultures showed no subcutaneous tumorigenic growth. Millions of cells per site were injected subcutaneously in *nude* mice. No palpable tumors were found after 3 months and there were no subcutaneous foci visible in postmortem examinations. Taken together, these observations indicate that the neurofibroma SC cultures, even the type-3 cultures, had low tumorigenic potential in these classical assays.

Neurofibroma SC Cultures Grafted in the Mouse Nerve

The tumorigenic growth of selected neurofibroma SC cultures was examined as xenografts in the sciatic nerves of adult immunodeficient *scid* mice. Each NF1 culture was engrafted into four nerves and six nerves were engrafted with an equal number of normal human SCs. Engrafted nerves were examined by immunostaining with an antibody specific to human glutathione S-transferase (Figure 6). First, transplantation of normal SCs resulted in transient occupancy (Figure 6C) and survival appeared to be severely limited because in four of six of the nerves glutathione S-transferase labeling was absent after 8 weeks. In contrast, all neurofibroma SC cultures (seven of seven) showed persistent and diffuse intraneural growth throughout the same period. Typically, neurofibroma SCs emanated from glutathione S-transferase-positive foci and grew in extensive longitudinal streams that intermingled with the host nerve elements (Figure 6A). Tumor cell migration also was associated with the nerve sheaths. Some NF1 SC cultures developed sizable masses that displaced nerve elements and caused significant regional enlargement of the nerve diameter (Figure 6B). Tumor masses varied in size for the different culture grafts, but additional work is required to quantitate the size and distribution of the tumor grafts. It will also be important to determine whether growth rates of the cultures, grafts, and originating tumors are related. Nevertheless, all observations indicated that tumor development in the mouse nerve, like that of human neurofibromas, was relatively slow and benign. There were only sporadic signs of functional impairment associated with the largest tumors and no mortality was associated with the transplants for up to 8 weeks. These results demonstrate reliable and sustained tumor growth by neurofibroma-derived human SCs implanted in the mouse nerve.
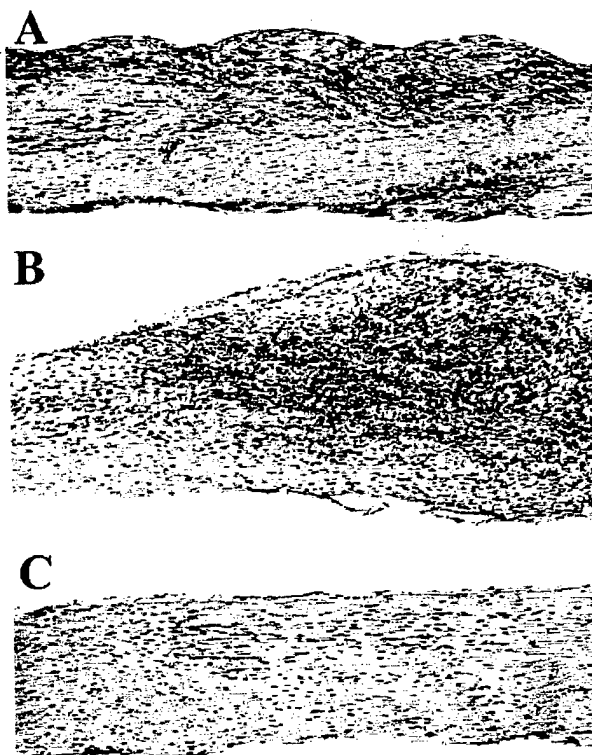
Figure 6. Tumor growth by human NF1 SC cultures xenografted into the nerves of *scid* mice. SC cultures from neurofibromas were transplanted into the nerves of immunodeficient mice. The growth and distribution of engrafted cells was traced by immunostaining with an antibody specific to human glutathione S-transferase. NF1 SC xenografts 4 weeks (**A**) and 8 weeks (**B**) after engraftment in the mouse sciatic nerve developed sizable masses and substantially increased the nerve diameter. NF1 SCs invaded along the longitudinal nerve axis between axons and along the epineurial sheath. Even the most extensive graft of normal SCs showed meager growth after 8 weeks, whereas most normal SC grafts failed to survive (**C**). Sections were counterstained with hematoxylin. Original magnification, ×100.

Discussion

In general, neurofibroma SCs, like their counterparts in normal nerves, fail to proliferate *in vitro* in the absence of specific mitogens.[5,7] Past efforts to establish models for human neurofibromas have relied on tissue explants and primary cultures of limited cell number with marked cellular heterogeneity. Only very recently have SCs been isolated from neurofibromas.[17–19] The lack of human neurofibroma-derived SC cultures and the low tumorigenic potential of neurofibroma cells in animal models has hampered the study of these prevalent tumors. Here we report the subculture of SCs from 10 dermal and eight plexiform NF1 tumors. All 18 of these NF1 SC cultures were found to be neurofibromin-deficient. Interestingly, many of the originating tumors contained both neurofibromin-negative and neurofibromin-positive SCs, indicating that SCs that lack functional *NF1* alleles have a distinct growth advantage *in vitro*. This advantage has been exploited by our subculture enrichment procedures using GGF-2 (without forskolin) and laminin.

Loss of *NF1* gene expression has been reported in malignant and benign SC tumors and pheochromocytomas from patients with NF1.[32,33] Based on the two-hit

hypothesis of tumor suppressor genes such as *NF1*, it is expected that neurofibromas should contain a supernumerary population of neurofibromin-deficient cells that are tumorigenic. Recent findings of cytogenetic alterations, loss of heterozygosity, and the absence of *NF1* mRNA expression in SCs cultured from neurofibromas strongly implicate SCs as the major neoplastic elements of dermal and plexiform neurofibromas.[17-19] In addition, in the present study we found that SCs isolated from numerous neurofibromas lack neurofibromin expression, confirming that *NF1* is inactivated in these SCs. Despite these persuasive findings, immunohistochemical evidence for a dominant population of neurofibromin-deficient cells has been highly ambiguous and scarcely reported. In an earlier study of numerous NF1 tumors, we concluded that a majority of neurofibromas consist mainly of SCs that express neurofibromin.[34] This antithetical observation was based on a large number of random archival specimens and, moreover, held true for a significant proportion of neurofibrosarcomas as well. However, on closer scrutiny, to examine the two-hit hypothesis at the single-cell level, it became evident that neurofibromin-negative SCs were present in most, if not all, neurofibromas. In the present study we focused on an independent group of neurofibromas from which SC subculture was successful. In most of the tumors in this group, neurofibromin labeling of tumoral elements was particularly sparse or absent. The remainder contained mixed populations of neurofibromin-negative and neurofibromin-positive SCs. The neurofibromin antibody used in these studies was raised against a peptide corresponding to amino acids 509 to 528 of the predicted *NF1* gene product. Directed against an N-terminal epitope, the antibody should bind to known neurofibromin splice variants as well as highly truncated (abnormal) forms. These results provide strong evidence that neurofibromin-negative cells of SC lineage contribute centrally to the formation of at least a significant subset of both dermal and plexiform neurofibromas. Nevertheless, there was notable variability in neurofibromin expression by SCs within many of the neurofibromas. The presence of neurofibromin expressing (*NF1* heterozygous) SCs suggests they too may perpetuate tumor formation, perhaps driven by the paracrine influence of the neurofibromin-deficient cell population. In this regard, Gutmann and co-workers[35] reported that neurofibromin expression by SCs in benign tumors may be down-regulated by factors produced within the tumor. Thus, paracrine influences may represent a novel mechanism for inactivating growth-suppressing genes and allowing for increased cell proliferation in tumors even in nonclonal cells. It is interesting that proliferation of neurofibromin-deficient SCs in response to GGF-2 was not enhanced by forskolin. Because forskolin increases SC expression of growth factor receptors including the GGF receptors erbB2 and erbB3,[36,37] this suggests that neurofibroma SCs express high levels of GGF receptors. Hyperexpression of erbB receptors has been reported in NF1 tumors and an inverse expression pattern of erbB2 and neurofibromin was shown for human SCs.[38,39] Additionally, SCs can express GGF and, at doses submaximal for proliferation, GGF-2

increases and directs the migration of SCs.[40-42] Taken together, these findings raise the possibility that GGF may function in an autocrine/paracrine mechanism that supports the continued growth of SCs in neurofibromas.

Despite considerable advances in the molecular genetics of NF1, the histogenesis of neurofibromas remains enigmatic. Dermal and plexiform neurofibromas contain a variety of cell types including SCs, perineurial cells, and fibroblasts. It is commonly held that despite their cellular complexity the histological features of neurofibromas are monotonously consistent.[43] In contradistinction, our studies of numerous neurofibromas and their derivative cell cultures indicate there are several levels of cellular and genetic diversity in this class of benign peripheral nerve sheath tumor. Cytogenetic abnormalities were identified in one of five of the dermal (our unpublished observation) and four of six of the plexiform SC cultures.[17] There were no consistent chromosomal regions involved in the abnormal karyotypes, suggesting that originating tumors are heterogeneous and may bear a variety of primary and/or secondary genetic changes. Additionally, the two plexiform cultures that displayed no cytogenetic rearrangements showed GGF-independent growth, suggesting that they either contain underlying genetic abnormalities not yet detected, or have expression abnormalities because of epigenetic influence. Clearly, neurofibromin deficiency did not confer GGF-2-independent growth on all of the developed SC cultures. Despite some differences in morphology, GGF-2 dependence, and karyotype, all NF1 SC cultures showed similarly low tumorigenic potential in several classical *in vitro* assays. However, the neurofibroma SC cultures showed a strong propensity to aggregate and form culture tumors. A similar growth pattern was observed for suspension cultures whereby NF1 SCs readily grew in aggregates reminiscent of tumor spheroids (an *in vitro* model for tumorigenic growth). Neurofibroma culture tumors contained an extensive laminin-rich extracellular matrix similar to that observed in neurofibromas.[30] These observations attest to a tumorigenic property of neurofibromin-deficient SC cultures that may be related to the adhesive mechanisms involved in the growth and development of neurofibromas. Sheela and co-workers[15] first demonstrated that NF1 SCs are angiogenic and invasive. In subsequent studies, which included two of the NF1 SC cultures used in the present report, we also concluded that NF1 SCs have a high invasive potential and a loss of negative autocrine growth control.[16] Despite these tumorigenic properties, our NF1 SC cultures, as well as the neurofibroma cultures used by Sheela and colleagues,[15] failed to form subcutaneous tumors in immunodeficient mice. Taken together, these findings imply that NF1 SCs have a tumorigenic potential that was not fully expressed in the *in vivo* model systems used previously.

Inceptive studies demonstrated the growth of implanted human neurofibroma tissue or SC preparations into the sciatic nerves of immunodeficient mice and the potential of this xenograft model for studying the tumorigenesis in NF1.[14,44] In the present study neurofibroma-like tumors resulted from the transplantation of neurofibromin-deficient NF1 SC cultures into the nerves of *scid*

mice. Extensive migration was consistently observed and many tumors were sizeable and substantially enlarged in the mouse nerve. There were apparent differences in the growth by the different transplanted NF1 SC cultures but overall tumor expansion was slow, suggesting that the growth rate of the developed tumors may reflect that of human neurofibromas. Additional studies are required to determine whether the growth patterns of the engrafted SC tumors correlate with those of the originating human tumors. This intraneural engraftment model is the first to achieve tumorigenic growth *in vivo* by human neurofibromin-deficient SCs and provides the means to study the histogenesis of neurofibromas in a relevant cellular environment. A further enhancement to this NF1 tumor model will be to transplant these neurofibroma SCs in the nerves of immunodeficient mice that are also heterozygous for *Nf1*. This highly relevant model of neurofibroma will also provide the opportunity to observe the interactions and contributions of engrafted ($NF1^{-/-}$) cells and ($Nf1^{-/+}$) nerve elements with the same genetic background as those found in NF1 patients.

Acknowledgments

References

1. Tonsgard JH, Kwak SM, Short P, Dachman AH: CT imaging in adults with neurofibromatosis-1—Frequent asymptomatic plexiform lesions. Neurology 1998, 50:1755–1760
2. Gutmann DH, Collins FS: Neurofibromatosis type 1. The Metabolic and Molecular Bases of Inherited Disease. Edited by CR Scriver, AL Beaudet, WS Sly, D Valle. New York, McGraw-Hill, 1995, pp 677–696
3. Peltonen J, Jaakkola S, Lebwohl M, Renvall S, Risteli L, Virtanen I, Uitto J: Cellular differentiation and expression of matrix genes in type 1 neurofibromatosis. Lab Invest 1988, 59:760–771
4. Erlandson RA, Woodruff JM: Peripheral nerve sheath tumors: an electron microscopic study of 43 cases. Cancer 1982, 49:273–287
5. Krone W, Mao R, Muhleck OS, Kling H, Fink T: Cell culture studies on neurofibromatosis (von Recklinghausen). Characterization of cells growing from neurofibromas. Ann NY Acad Sci 1986, 486:354–370
6. Atit RP, Crowe MJ, Greenhalgh DG, Wenstrup RJ, Ratner N: The Nf1 tumor suppressor regulates mouse skin wound healing, fibroblast proliferation, and collagen deposited by fibroblasts. J Invest Dermatol 1999, 112:835–842
7. Sobue G, Sonnenfeld K, Rubenstein AE, Pleasure D: Tissue culture studies of neurofibromatosis: effects of axolemmal fragments and cyclic adenosine 3′,5′-monophosphate analogues on proliferation of Schwann-like and fibroblast-like neurofibroma cells. Ann Neurol 1985, 18:68–73
8. Nakamura T, Hara M, Kasuga T: Early lesion of N-nitroso-N-ethylurea-induced hamster neurofibroma model. Cancer Res 1991, 51:434–438
9. Kharbanda K, Dinda AK, Sarkar C, Karak AK, Mathur M, Roy S: Cell culture studies on human nerve sheath tumors. Pathology 1994, 26:29–32
10. Feany MB, Anthony DC, Fletcher CDM: Nerve sheath tumours with hybrid features of neurofibroma and schwannoma: a conceptual challenge. Histopathology 1998, 32:405–410
11. Colman SD, Williams CA, Wallace MR: Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the NF1 gene. Nat Genet 1995, 11:90–92
12. Brannan CI, Perkins AS, Vogel KS, Ratner N, Nordlund ML, Reid SW, Buchberg AM, Jenkins NA, Parada LF, Copeland NG: Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev Rev 1994, 8:1019–1029
13. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet 1994, 7:353–361
14. Lee JK, Sobel RA, Chiocca EA, Kim TS, Martuza RL: Growth of human acoustic neuromas, neurofibromas and schwannomas in the subrenal capsule and sciatic nerve of the nude mouse. J Neurooncol 1992, 14:101–112
15. Sheela S, Riccardi VM, Ratner N: Angiogenic and invasive properties of neurofibroma Schwann cells. J Cell Biol 1990, 111:645–653
16. Muir D: Differences in proliferation and invasion by normal, transformed and NF1 Schwann cell cultures are influenced by matrix metalloproteinase expression. Clin Exp Metastasis 1995, 13:303–314
17. Wallace MR, Rasmussen SA, Lim IT, Gray B, Zori RT, Muir D: Culture of cytogenetically abnormal Schwann cells from benign and malignant NF1 tumors. Genes Chromosom Cancer 2000, 27:117–123
18. Kluwe L, Friedrich R, Mautner VF: Loss of NF1 allele in Schwann cells but not in fibroblasts derived from an NF1-associated neurofibroma. Genes Chromosom Cancer 1999, 24:283–285
19. Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E: Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. Hum Mol Genet 2000, 9:1059–1066
20. Rutkowski JL, Tennekoon GI, McGillicuddy JE: Selective culture of mitotically active human Schwann cells from adult sural nerves. Ann Neurol 1992, 31:580–586
21. Casella CTB, Bunge RP, Wood PM: Improved method for harvesting human Schwann cells from mature peripheral nerve and expansion in vitro. Glia 1996, 17:327–338
22. Bottenstein JE, Sato GH: Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc Natl Acad Sci USA 1979, 76:514–517
23. Muir D: Metalloproteinase-dependent neurite outgrowth within a synthetic extracellular matrix is induced by nerve growth factor. Exp Cell Res 1994, 210:243–252
24. Gutmann DH, Aylsworth A, Carey JC, Korf B, Marks J, Pyeritz RE, Rubenstein A, Viskochil D: The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. JAMA 1997, 278:51–57
25. Neugut AI, Weinstein IB: Use of agarose in the determination of anchorage-independent growth. In Vitro 1979, 15:351–358
26. Muir D, Varon S, Manthorpe M: An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. Anal Biochem 1990, 185:377–382
27. Gutmann DH, Wood DL, Collins FS: Identification of the neurofibromatosis type 1 gene product. Proc Natl Acad Sci USA 1991, 88:9658–9662
28. Zorick TS, Lemke G: Schwann cell differentiation. Curr Opin Cell Biol 1996, 8:870–876
29. Rutkowski JL, Kirk CJ, Lerner MA, Tennekoon GI: Purification and expansion of human Schwann cells in vitro. Nat Med 1995, 1:80–83
30. Leivo I, Engvall E, Laurila P, Miettinen M: Distribution of merosin, a laminin-related tissue-specific basement membrane protein, in human Schwann cell neoplasms. Lab Invest 1989, 61:426–432
31. Daston MM, Scrable H, Nordlund M, Sturbaum AK, Nissen LM, Ratner N: The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells, and oligodendrocytes. Neuron 1992, 8:415–428
32. Takahashi K, Suzuki H, Hatori M, Abe Y, Kokubun S, Sakurai M, Shibahara S: Reduced expression of neurofibromin in the soft tissue tumours obtained from patients with neurofibromatosis type 1. Clin Sci 1995, 88:581–585
33. Gutmann DH, Cole JL, Stone WJ, Ponder BA, Collins FS: Loss of

neurofibromin in adrenal gland tumors from patients with neurofibromatosis type I. Genes Chromosom Cancer 1994, 10:55–58

34. Rojiani AM, Rojiani MV, Wallace MR, Johnson J, Muir D: Expression of the NF1 gene product in peripheral nerve sheath tumors from NF1 patients. J Neuropathol Exp Neurol 1999, 58:524

35. Gutmann DH, Silos-Santiago I, Geist RT, Daras M, Rutkowski JL: Lack of NF1 expression in a sporadic schwannoma from a patient without neurofibromatosis. J Neurooncol 1995, 25:103–111

36. Weinmaster G, Lemke G: Cell-specific cyclic AMP-mediated induction of the PDGF receptor. EMBO J 1990, 9:915–920

37. Cheng LL, Esch FS, Marchionni MA, Mudge AW: Control of Schwann cell survival and proliferation: autocrine factors and neuregulins. Mol Cell Neurosci 1998, 12:141–156

38. Rowley PT, Kosciolek B, Bader JL: Oncogene expression in neurofibromatosis. Ann NY Acad Sci 1986, 486:327–332

39. Schlegel J, Muenkel K, Trenkle T, Fauser G, Ruschoff J: Expression of the ERBB2/neu and neurofibromatosis type 1 gene products in reactive and neoplastic schwann cell proliferation. Int J Oncol 1998, 13:1281–1284

40. Raabe TD, Clive DR, Neuberger TJ, Wen D, DeVries GH: Cultured neonatal Schwann cells contain and secrete neuregulins. J Neurosci Res 1996, 46:263–270

41. Carroll SL, Miller ML, Frohnert PW, Kim SS, Corbett JA: Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. J Neurosci 1997, 17:1642–1659

42. Mahanthappa NK, Anton ES, Matthew WD: Glial growth factor 2, a soluble neuregulin, directly increases Schwann cell motility and indirectly promotes neurite outgrowth. J Neurosci 1996, 16:4673–4683

43. Riccardi VM: Neurofibromatosis: Phenotype, Natural History, and Pathogenesis, ed 2. Baltimore, Johns Hopkins University Press, 1992

44. Appenzeller O, Kornfeld M, Atkinson R, Snyder RD: Neurofibromatosis xenografts. Contribution to pathogenesis. J Neurol Sci 1986, 74:69–77

UNIVERSITY OF FLORIDA

MEMORANDUM

DATE: May 23, 2001

TO: Margaret R. Wallace, Ph.D.
           Box 100266

FROM: R. Peter Iafrate, Pharm.D.
           Chair, IRB-01

SUBJ: IRB Protocol #41-1992   **Expires on 6/2/02**

TITLE: "GENETIC STUDIES OF NEUROFIBROMATOSIS 1" [SUBTITLE: GENETIC STUDIES OF TUMORIGENESIS IN NEUROFIBROMATOSIS] [SUBTITLE: THE ROLE OF CUMULATIVE GENETIC DEFECTS IN NF1 TUMORIGENESIS] [SUBTITLE: MUTATION AND GENETIC ANALYSIS OF NEUROFIBROMATOSIS]

Re-approval of this research project was granted on 5/16/01. Enclosed is the dated, **IRB-approved Informed Consent Form which must be used for enrolling subjects into this project from 6/2/01 - 6/1/02.**

You are responsible for obtaining renewal of this approval prior to the expiration date. Re-approval of this project must be granted before the expiration date or the project will be automatically suspended. If suspended, new subject accrual must stop. Research interventions must also stop unless there is a concern for the safety or well being of the subjects. **You must respond to the continuing review questions within 90 days or your project will be officially terminated.**

The IRB has approved exactly what was submitted. Any change in the research, no matter how minor, may not be initiated without IRB review and approval, except where necessary to eliminate hazards to human subjects. If a change is required due to a potential hazard, that change must be promptly reported to the IRB.

Any severe or unanticipated side effects or problems, and all deviations from federal, state, university or IRB regulations must be reported, in writing, within 5 working days.

Upon completion of the study, you are required to submit a summary of the project to the IRB office.

Research records must be retained for three years after completion of the research; if the study involves medical treatment, it is recommended that the records be retained for eight years.

If VAMC patients will be included in this project, or if the project is to be conducted in part on VA premises or performed by a VA employee during VA-compensated time, review by the VA Subcommittee for Research is required.

You are responsible for notifying all parties about the approval of this project, including your co-Investigators and Department Chair. If you have any questions, please feel free to contact the IRB-01 office at (352) 846-1494.

Cc: IRB File
       Pharmacy
       VA Research Center
       Clinical Research Center